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(54) Title: METHODS AND COMPOSITIONS FOR HIGH PROTEIN PRODUCTION FROM RECOMBINANT DNA (57) Abstract <p>Disclosed herein are improved methods and compositions for achieving enhanced protein production expressed from non-native gene constructs, including single chain sFv and derivative sequences. The methods and compositions are particularly useful for creating stably transfected, constitutively expressing immortalized mammalian cell lines that exhibit high recombinant protein productivity while maintaining a low copy number per cell of the non-native recombinant DNA sequence encoding the protein of interest.</p>		

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**"METHODS AND COMPOSITIONS FOR HIGH PROTEIN
PRODUCTION FROM RECOMBINANT DNA"**

FIELD OF THE INVENTION

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The application relates generally to the field of recombinant gene expression.

BACKGROUND OF THE INVENTION

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Improved methodologies for maximizing protein production through recombinant gene expression is an on-going effort in the art. Of particular interest is the development of methodologies that maximize
15 recombinant expression of biologically active proteins for producing commercially useful quantities of these proteins. While prokaryotic, typically bacterial, host cell systems have proven capable of generating large quantities of recombinant proteins, these hosts suffer
20 from a number of disadvantages, including an inability to glycosylate proteins, inefficient cleavage of "pre" or "prepro" sequences from proteins (e.g., inefficient post translational modification), and a general inability to secrete proteins. Consequently the art
25 has sought eukaryotic host systems, typically mammalian host cell systems, for mammalian protein production. One feature of such systems is that the protein produced has a structure most like that of the natural protein species, and, purification often is easier
30 since the protein can be secreted into the culture medium in a biologically active form.

A number of problems still exist however, in mammalian culture systems. Specifically, high levels
35 of production typically are not easily obtained in mammalian systems. In addition, eukaryotic host cells typically have more stringent requirements for

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culturing and have slower growth rates. Thus, producing large quantities of a recombinant protein requires more than simply culturing a host cell transfected with an expression vector. This is particularly true when the gene of interest encodes a protein that is poorly expressed, e.g., is not produced in abundance and/or is only transiently produced under natural, physiological conditions. Typically, the genes for these proteins have multiple levels of regulation, often at one or more levels of the expression system, e.g., at the level of transcription, translation, post translation modification, secretion and/or activation. Typically these genes, when stably integrated in unamplified, immortalized cells, produce less than about 10-100 ng protein/ 10^6 cells/ml. Maximizing production of these proteins means identifying means for circumventing these levels of regulation.

One approach to achieving enhanced protein production is use of transient cell expression systems wherein cells are transfected with high copy numbers of plasmids that are not expected to integrate in the host cell genome. The plasmids used in transient cell expression systems also can be modified to further enhance their copy numbers during replication post transfection. While the transfection event typically limits the life of these cells to only several generations, reasonable quantities of the desired protein may be produced while the cells remain alive. Because such transient cell systems are short-lived they are not cell systems of choice for commercial production systems. Transient cell systems often are used to screen candidate plasmid or other vector constructions as part of the development of an immortalized, constitutive cell line. But, because transient expression systems are short lived, the

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long-term productivity of a particular vector construction (or its effect, once integrated, on the viability of a cell after many generations) can not be determined with certainty. Accordingly, a number of
5 plasmid constructions, while productive in transient cell systems, have been determined not to be useful in established cell lines, an event that generally cannot be determined until an established cell line is created.

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Two alternative ways primarily focused on by the art for enhancing recombinant gene expression in eukaryotic host systems are enhancing the gene copy number, typically by gene amplification, and enhancing
15 the efficiency of expression of each gene copy. The most common method for enhancing gene copy number is by selecting for gene amplification wherein the host cell is transformed with two genes, linked or unlinked, one of which encodes the desired protein and the other of
20 which encodes an amplifiable selectable marker, such as dihydrofolate reductase (DHFR.) Transformed cells then are cultured in the presence of increasing concentrations of a toxic agent (e.g., methotrexate, where the amplifiable marker is DHFR) whose effects can
25 be nullified by expression of the selectable marker gene. In response to high concentrations of the toxic agent cells survive because they have amplified the copy number of the selectable marker gene and, fortuitously, the desired protein gene. Using this
30 methodology copy numbers in the hundreds and thousands/cell have been achieved.

While gene amplification has proven to be useful, the methodology suffers from several disadvantages
35 pertinent to commercial production. For example, the production of a highly productive cell line by gene amplification alone, e.g., having thousands of copies

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of the gene of interest, is a time-consuming process often requiring between 6-10 months to complete. Moreover, at very high copy number, verification of the nucleotide sequence integrity for each gene copy in a cell is difficult or not possible. Accordingly, point mutations and other sequence modifications that can alter the biological activity of the protein product may not be detected, and further may pose problems with compliance of government (e.g., FDA) regulations. Moreover, maintenance of such a high copy number requires maintaining the selective pressure by maintaining high levels of the toxic agent in the culture medium. This is both expensive and presents additional regulatory issues when purifying the protein of interest from the culture medium. Finally, and perhaps most importantly, when a gene has multiple levels of expression regulation, merely increasing the copy number of the DNA may not be sufficient to enhance protein production significantly.

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One method for enhancing recombinant DNA expression is by means of one or more genes encoding expression effector molecules. Among the effector molecules known in the art are transacting transcription activators which can stimulate transcription of heterologous genes. Examples include the simian virus (SV40) T antigen and adenovirus E1A and E1B proteins which can act on certain viral promoters of heterologous genes, including the cytomegalovirus (CMV) major intermediate early (MIE) promoter. Other molecules reported to have this transactivating activity include the immediate early (IE) proteins of herpes virus, C-myc and genes of the human and simian acquired immunodeficiency virus.

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Other viral genes which can effect mammalian protein production are viral translational control effectors. Examples include RNA sequences encoded by

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the adenovirus, such as the VA genes (VA1 and VA2). Such sequences are believed to assist protein production by assisting with translation initiation, probably by association with one or more translation
5 initiation factors. Other sequences include RNA sequences that can enhance stability of the mRNA transcript.

Cockett et al., ((1990) Nucleic Acids Research
10 19:319-325 and EP application 378,382) describe the use of the adenovirus E1A genes as an alternative to gene amplification for recombinant protein expression in Chinese hamster ovary (CHO) cells, where the gene of interest is under the CMV promoter control. The level
15 of protein produced is asserted to approach levels achievable by gene amplification, thereby obviating the need for gene amplification. Moreover, the authors see no substantial increase in protein productivity when the E1A gene is introduced to an amplified cell line
20 expressing the gene of interest.

U.S. Patent No. 5,024,939 describes an unamplified transient cell expression system producing "useful" quantities of a desired gene product in 1 to 14 days
25 without having to establish a continuous production cell system. The authors transfect E1A-expressing cells ("293" cells) with a large number of plasmids carrying the gene of interest under CMV promoter control, and demonstrate increased protein production
30 in these cells for the short lives of the cells. Co-transfection of the 293 cells with the adenovirus VA1 gene appears to double the amount of protein produced in these cells.

35 It is an object of the instant invention to provide a method for enhancing protein production of poorly expressed genes by recombinant DNA technology. It is

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another object of the invention to provide immortalized cell lines suitable for commercial exploitation wherein the cells are stably transfected with the gene of interest and are competent to constitutively express
5 the gene of interest, and methods for producing these cell lines. Still another object of the invention is to provide cell lines and methods for creating them, exhibiting high recombinant protein productivity while maintaining a low copy number per cell of the
10 recombinant DNA sequences encoding the protein. Yet another object is to provide cell lines that can be adapted to grow in low serum or serum-free medium.

Importantly, it is another object of the instant
15 invention to provide means for producing commercially-feasible quantities of morphogenic proteins from cultures of immortalized, stably transfected CHO cell lines.

20 These and other objects and features of the invention will be apparent from the description, drawings, and claims which follow.

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SUMMARY OF THE INVENTION

An improvement in recombinant protein production methodologies now has been discovered which has particular application for the expression of "low expression" or "poorly expressing" genes. As a result of this invention, commercial scale production quantities of hard-to-produce proteins now can be obtained from stably transfected, constitutively expressing eukaryotic cells. Moreover, the cell lines taught by this invention exhibit high recombinant protein productivity while maintaining a low copy number per cell of the recombinant DNA sequences encoding the protein. The cell lines of the invention also can be adapted to grow in low serum or even serum-free medium without significantly compromising cell growth or protein productivity.

The invention involves the multiple transfection of an immortalized eukaryotic cell with a gene of interest and at least one, and preferably, two expression effector genes of viral origin competent to effect expression of the gene of interest, culturing the transfected cell under appropriate selection conditions such that the transfected DNA is stably integrated into the cell genome, and selecting a clone that expresses at least 1 μg protein/ 10^6 cells/ml at post logarithmic phase, for cells grown in a "batch" or "terminal" cell culture. In a preferred embodiment, the clone expresses at least 5 μg protein/ 10^6 cells/ml, or at least 10 μg protein/ 10^6 cells/ml. As will be appreciated by those having ordinary skill in the art, higher protein productivity can be obtained by modifying culturing conditions, for example, to enhance cell growth or cell number. In another preferred embodiment, the gene of interest is cotransfected with a means for amplifying the gene, and the cell is

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cultured under selection conditions that induce gene amplification. While any means for gene amplification is contemplated to be useful, the currently preferred means of gene amplification is by cotransfection of a
5 gene encoding an amplifiable selection marker, such as for example, DHFR or adenosine deaminase, in operational association with a transcription unit. Most preferably, the amplifiable selection marker gene is on the same nucleic acid or vector that carries the
10 gene of interest.

While the method of the invention is described with reference to a single cell, as will be appreciated by those having ordinary skill in the art, this is only
15 for ease of description and the method is most efficiently carried out using a plurality of cells.

As used herein, "vector" is understood to mean any nucleic acid comprising a nucleotide sequence of
20 interest and competent to be incorporated into a host cell and recombining with and integrating into the host cell genome. Such vectors include linear nucleic acids, plasmids, phagemids, cosmids and the like.

25 As used herein, "gene expression" is understood to refer to the production of the protein product encoded by a DNA sequence of interest, including the transcription of the DNA sequence and translation of the mRNA transcript.

30

As used herein, "poorly expressed genes" is understood to describe genetic sequences, e.g., DNA sequences that can be acted on by an RNA polymerase to produce an mRNA transcript, and which are not easily
35 expressed and for which only low levels, e.g., less than 10-100 ng protein/ 10^6 cells/ml are produced in an unamplified, stably integrated immortalized eukaryotic

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host cell system, and for which less than about 100-1000 ng protein/ 10^6 /ml are produced in amplified cell. For example, a highly amplified eukaryotic cell is a transfected cell subcloned sufficiently to contain
5 about 1000 or more copies of the gene of interest stably integrated into the host cell's genome and in operative association with a strong promoter/enhancer unit.

10 Typically, examples of poorly expressed genes are genes whose expression is highly regulated under naturally occurring conditions. Examples of such genes include protein hormones, Factor VIII, TPA (tissue plasminogen activator), and the class of proteins
15 called tissue morphogens or morphogenic proteins(see, for example, PCT/US92/07432 (WO93/05751); or PCT/US93/08808.) Poorly expressed genes are characterized as being highly regulated at one or more levels of expression, e.g., at the level of
20 transcription, translation, post translational modification, secretion and/or protein activation.

Another class of genes for which substantial protein production is difficult to obtain include non-
25 native, biosynthetic or otherwise artificial genes, such as genes created by rational design, and which contain one or more non-native DNA and/or RNA sequences or structures with which the host expression system is unfamiliar and which may limit or otherwise interfere
30 with efficient protein production. An example of such an artificial sequence which does not occur in nature is the single chain binding site molecule (also referred to in the art as "BABS," "biosynthetic antibody binding sites" molecules) wherein a light and
35 heavy chain are encoded in a single DNA sequence, linked by a sequence encoding a polypeptide linker (see, for example, U.S. Patent Nos. 5,132,405 and

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5,091,513). To date, it is not certain what the limiting step or steps in efficient expression of these genes may be; such limitations may include inefficient secretion.

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In a preferred embodiment of the invention the gene of interest encodes a member of the class of proteins called morphogenic proteins as defined in USSN 08/091,395 and in PCT/US92/01968 (WO92/15323) or PCT/US92/07432 (WO93/05751) or PCT/US93/08808, the disclosures of which are incorporated herein by reference. These morphogenic proteins contemplated by the invention include, but are not limited to, the group consisting of OP1, OP2, OP3, BMP2, BMP3, BMP4, BMP5, BMP6, BMP9, DPP, Vgl, Vgr, 60A protein, GDF-1, GDNF, dorsalin-1, and amino acid sequence variants thereof which do not alter substantially the morphogenic activity of these proteins in vivo. As defined in these specifications the members of this class of proteins are characterized as competent for inducing the developmental cascade of cellular and molecular events that culminate in the formation of new organ-specific tissue, including any vascular and connective tissue formation as required by the naturally occurring tissue. Specifically, the morphogens are competent for inducing all of the following biological functions in a morphogenically permissive environment: (1) stimulating proliferation of progenitor cells; (2) stimulating differentiation of progenitor cells; (3) stimulating the proliferation of differentiated cells and (4) supporting the growth and maintenance of differentiated cells. In a particular embodiment, the proteins can induce the full developmental cascade of bone tissue morphogens, including the migration and proliferation of mesenchymal cells, proliferation and differentiation of chondrocytes, cartilage matrix formation and

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calcification, vascular invasion, osteoblast proliferation, bone formation, bone remodeling, and hematopoietic bone marrow differentiation.

5 The genetic sequences encoding these proteins and descriptions for their isolation from various genomes are disclosed in the art as follows: OP1 (U.S. 5,011,691; Ozkaynak et al. (1990) EMBO J. 9: 2085-2093 and USSN 07/841,646, filed February 21, 10 1992, soon to be issued as US Patent No. ____; OP2 (Ozkaynak (1992) J. Biol. Chem. 267:25220-25227 and USSN 07/841,646); BMP 2,3,4 (Wozney et al. (1988) Science 242:1528-1534); BMP5,6 (Celeste et al. (1991) PNAS 87:9843-9847); BMP 9 (WO93/00432, published 15 January 7, 1993); GDF-1 (Lee (1991) PNAS 88:4250-4254); DPP (Padgett et al. (1987) Nature 325:81-84); Vg-1 (Weeks (1987) Cell 51:861-867); Vgr-1 (Lyons et al. (1989) PNAS 86:4554-4558); 60A (Wharton et al. (1991) PNAS 88:9214-9218); GDNF (Lin et al. (1993) Science 20 260: 1130-1132) and Dsl-1 (dorsalin-1, Basler et al. (1993) Cell 73: 687-702), the disclosures of which are incorporated herein by reference.

25 The genetic sequences encode proteins the immature translation products of which comprise a secretion signal sequence and a "pro" domain, both of which are cleaved to release polypeptide chains of about 135-145 amino acids, depending on the protein species. The members of the class are characterized in 30 part by significant amino acid homology (e.g., at least 70% homology) within the C-terminal 102-106 amino acids, including seven cysteines, substantially conserved in their linear arrangement in the C-terminal sequence.

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 The proteins typically are secreted as disulfide linked dimers and are rendered soluble under physiological conditions by non-covalent association

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with one or more copies of the cleaved pro domain. A detailed description of these proteins and their physiological forms is disclosed in USSN 08/040,510, March 31, 1993, the disclosure of which is incorporated
5 herein by reference.

The morphogens are inactive when reduced, but are active as oxidized homodimers and when oxidized in combination with other morphogens of this invention.
10 Thus, as defined herein, a morphogen is a dimeric protein comprising a pair of polypeptide chains, wherein each polypeptide chain comprises at least the C-terminal six cysteine skeleton defined by the C-terminal 96 amino acids of the mature OP1 polypeptide
15 sequence, including functionally equivalent arrangements of these cysteines (e.g., amino acid insertions or deletions which alter the linear arrangement of the cysteines in the sequence but not their relationship in the folded structure), such that,
20 when the polypeptide chains are folded, the dimeric protein species comprising the pair of polypeptide chains has the appropriate three-dimensional structure, including the appropriate intra- or inter-chain disulfide bonds such that the protein is
25 morphogenically competent in vivo.

Particularly useful sequences for use as morphogens include the C-terminal domains of the class morphogenic proteins, e.g., the C-terminal 96-102 amino
30 acid residues of Vgl, Vgr-1, DPP, OP-1, OP-2, BMP-2, BMP-4, GDF-1, GDNF, Dsl-1, 60A protein, BMP3, BMP5 BMP6 and BMP9, all of which include at least the conserved six or seven cysteine skeleton. In addition, biosynthetic constructs designed from the generic
35 sequences, such as COP-1, 3-5, 7, 16, disclosed in U.S. Pat. No. 5,011,691, also are useful. Other sequences include the inhibins/activin proteins (see, for

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example, U.S. Pat. Nos. 4,968,590 and 5,011,691).

Accordingly, other useful sequences are those sharing at least 70% amino acid sequence homology or "similarity", and preferably 80% homology or similarity with any of the sequences above. These are anticipated to include allelic, species variants and other amino acid sequence variants (e.g., including "muteins" or "mutant proteins"), whether naturally-occurring or biosynthetically produced, as well as novel members of this morphogenic family of proteins. As used herein, "amino acid sequence homology" is understood to mean amino acid sequence similarity, and homologous sequences share identical or similar amino acids, where similar amino acids are conserved amino acids as defined by Dayoff et al., Atlas of Protein Sequence and Structure; vol.5, Suppl.3, pp.345-362 (M.O. Dayoff, ed., Nat'l BioMed. Research Fdn., Washington D.C. 1978.) Thus, a candidate sequence sharing 70% amino acid sequence homology with a reference sequence requires that, following alignment of the candidate sequence with the reference sequence, 70% of the amino acids in the candidate sequence are identical to the corresponding amino acid in the reference sequence, or constitute a conserved amino acid change thereto. "Amino acid sequence identity" is understood to require identical amino acids between two aligned sequences. Thus, a candidate sequence sharing 60% amino acid identity with a reference sequence requires that, following alignment of the candidate sequence with the reference sequence, 60% of the amino acids in the candidate sequence are identical to the corresponding amino acid in the reference sequence.

As used herein, all homologies and identities calculated use OP-1 as the reference sequence. Also as used herein, sequences are aligned for homology and

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identity calculations using the method of Needleman et al. (1970) J.Mol. Biol. 48:443-453 and identities calculated by the Align program (DNASTar, Inc.) In all cases, internal gaps and amino acid insertions in the candidate sequence as aligned are ignored when making the homology/identity calculation.

The currently preferred protein sequences useful as morphogens include, but are not limited to, those having greater than 60% identity, preferably greater than 65% identity, with the amino acid sequence defining the conserved six cysteine skeleton of human OP1 (e.g., the C-terminal 96 amino acids). These most preferred sequences include both allelic and species variants, e.g., naturally-occurring sequence variants, of the OP-1 and OP-2 proteins, including the Drosophila 60A protein.

The expression effector molecules useful in the methods and cell lines of the invention preferably are of viral origin and are competent to stimulate transcription and translation. In one embodiment the expression effector molecules of viral origin are encoded in the bovine papilloma virus early region DNA (See Maat, J. et al (1979) Gene 6:75 et seq. and Molecular Cloning: A Laboratory Manual, 2ed. Sambrook et al. eds., Cold Spring Harbor Laboratory Press, NY (1989), Chapter 16, for a description of this region of the virus.)

30

In another embodiment, the expression effector molecules of viral origin include trans-acting transcription activators ("transcription transactivators") competent to act on and stimulate the promoter that induces transcription of the gene of interest. Typically, these transactivators are of

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viral origin and can act on their own or on other particular viral promoters. In a preferred embodiment, the transactivating protein is encoded by the adenovirus E1A or E1B gene, the herpesvirus IE gene, C-myc, or the TAT gene of the human immunodeficiency virus (HIV). For example, where the transactivating protein is E1A, the preferred promoters include the constitutive CMV-MIE promoter, and the adenovirus E1A and late region promoters. Where the transactivator is encoded by the TAT gene, a preferred promoter is the HIV-LTR. Other transactivator-promoter combinations are described in the art and are contemplated herein. As described in more detail below, the viral transcription activator gene need not be under the control of a promoter which limits its expression, but such a promoter may be employed.

In another preferred embodiment, the expression effector molecules of viral origin also include RNA sequences operative to promote translation of the transcript encoded by the gene of interest. These sequences may include mRNA stabilizing sequences or segments which act on the translation machinery itself. For example, currently most preferred sequences are those encoded by the adenovirus, most particularly the adenovirus VA genes, including VA1 and VA2. These genes encode RNAs that are thought to act, at least in part, by interaction with one or more translation initiation factors. The bovine papilloma virus early region DNA is anticipated to include one or more of these stabilizing sequences.

Preferably, the transfection system comprises both a gene encoding a transcription transactivating sequence and a gene encoding an RNA stabilizing sequence that stimulates translation.

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In another preferred embodiment, the transfected cells are subcloned under selective pressure to induce amplification of the gene of interest. The currently preferred method includes the use of a gene encoding an amplifiable selection marker. An example of such a marker gene used with success in the method of the invention includes the DHFR gene, and selection with methotrexate. However, other amplifiable genes are well known in the art and are contemplated herein, including, without limitation, adenosine deaminase and glutamine synthetase. A general description of gene amplification and useful selectable marker genes are described in a number of texts available in the art, including R.E. Kellems, Gene Amplification in Mammalian Cells, Marcel Dekker, New York (1993). Where the amplifiable selection gene is not dominant acting, the host cell to be transfected preferably is genotypically deficient in the selection gene.

Host cell lines contemplated to be useful in the method of the invention include any eukaryotic cell lines that can be immortalized, i.e., are viable for multiple passages, (e.g., greater than 50 generations), without significant reduction in growth rate or protein production. Where cell lines are to be used to produce biologicals intended for administration to humans, the host cell preferably is not a human cell. Currently preferred cell lines are those having simple media component requirements, and which can be adapted for suspension culturing. Most preferred are mammalian cell lines that can be adapted to growth in low serum or serum-free medium. Particularly, where the gene of interest encodes a morphogenic protein, the preferred host cell line is a mammalian tissue cell line, e.g., a uro-genital cell line including kidney or bladder cell line, liver, lung, ovary, cardiac muscle, or other smooth muscle cell line, including a smooth muscle cell

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line of the gastrointestinal tract. Representative cell lines include, but are not limited to, Chinese hamster ovary (CHO); canine kidney (MDCK); or rat bladder (NBT-2), and the like. Useful cell lines can
5 be obtained from the American Type Culture Collection (ATCC), Rockville, MD or from the European Collection of Animal Cell Cultures, Porton Down, Salisbury SP40JG, U.K.

10 Where the gene of interest is a "low expression" or "poorly expressed" gene, the currently most preferred methodology includes co-transfection of the gene of interest, the transactivating gene and the RNA stabilizing sequence, and subcloning candidate cells
15 under amplification conditions so as to produce a cell line that produces at least 1 μg protein/ 10^6 cells/ml, more preferably at least 5 μg protein/ 10^6 cells/ml, or at least 10 μg / 10^6 cells/ml in a "batch" or "terminal" cell culture where the protein is harvested from the
20 culture medium when the cells are in post-logarithmic phase.

An important feature of the invention is that the method of manufacturing a transfected host cell
25 line provides a low copy number of the gene of interest while still producing high levels of the protein product. This feature endows the invention with regulatory utility by easing the burden of compliance with federal good manufacturing practices. For example,
30 low copy number in the transfected cell line enabled and disclosed herein will permit ease of documentation and standardization of production methodologies pursuant to U.S. Food and Drug Administration rules and regulations.

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With respect to the transfection process used in the practice of the invention, all means for introducing nucleic acids into a cell are contemplated

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including, without limitation, CaPO_4 co-precipitation, electroporation, DEAE-dextran mediated uptake, protoplast fusion, microinjection and lipofusion. A key to the invention is the complement of vectors with
5 which the cell is transfected, rather than the mechanical or chemical process by which the DNA incorporation is accomplished.

Moreover, the invention contemplates either
10 simultaneous or sequential transfection of the host cell with vectors containing the DNA sequences to be integrated into the genome. In one preferred embodiment, host cells are simultaneously transfected with at least two unlinked vectors, one of which
15 contains the gene of interest (also referred to as the "reporter gene"), and the other of which contains a gene encoding a transcription transactivator. More preferably, genes encoding an amplifiable selection gene, and a translation stimulating sequence also are
20 cotransfected, either by incorporation of these sequences on one or both of the two unlinked vectors, or by simultaneous transfection with a third vector, followed by early transfectant selection based on cell growth and enhanced protein production. Simultaneous
25 transfection permits for random assortment of the genes to be incorporated into the host cell and allows the cells independently to regulate the copy number and expression level of the transfecter sequence. Thus, the final optimal combination is determined empirically
30 for each cell, in essence by each cell, by selecting for high protein producing cells that are also healthy, stable transfectants. The exact copy number of the gene elements and/or expression control elements for each gene's expression may vary among the clones
35 selected, but all are characterized by producing at least 1 μg protein per ml per 10^6 cells at post-logarithmic phase in a terminal cell culture. In a

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preferred embodiment, the cells also are characterized by having a low copy number of the gene of interest which may be due, at least in part, to the presence of the transcription transactivator.

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While there is no reason a priori why all elements cannot be transfected on a single vector, as will be appreciated by those skilled in the art, a single vector limits the possible constellations of the elements on the vector and, therefore, in the cell, rather than allowing for their random assortment in cells. Where all elements, e.g., the gene of interest, the amplifiable marker and the expression effector sequences are transfected on a single vector, the transactivating transcription effector gene preferably is under control of a weakened promoter to limit the expression of this gene sequence.

Alternatively, the DNA sequences can be transfected sequentially. For example, the vector comprising the transcription activator, e.g., E1A, may be transfected first, and its DNA allowed to stably integrate within the host cell genome prior to subsequent transfection with the remaining sequence(s). Also contemplated in the invention is the use of the expression effector genes under weak or strong promoter/enhancer units.

A key to realizing the benefit of the instant invention's enhanced production of poorly expressed genes is culturing the above-described transfected cell lines in low serum or serum-free medium. The currently preferred serum-free medium is a lipid-modified medium wherein the modification comprises a lipid membrane phosphoglyceride ester degradation product. A representative formulation of the preferred serum-free

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medium is presented in U.S.S.N. 07/124,676 (filed September 22, 1993) and incorporated herein by reference. Other media, including other serum free media, are described in the art.

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Thus, in view of this disclosure, skilled genetic engineers can construct transfectants which overcome the production problems associated with certain low expression genes. Specifically, those skilled in recombinant DNA techniques can design appropriate DNA vectors encoding for the protein of interest, an amplifiable marker gene, transcription transactivators, and translation stimulators, and then use the methods of manufacturing transfectants disclosed herein to obtain large quantities of proteins. Such proteins can be in their native forms or truncated analogs, as well as muteins, fusion proteins, or other constructs capable of mimicing the biological activity of the protein of interest in vivo.

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BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects and features of the invention, as well as the invention itself, may be
5 more fully understood from the following description, when read together with the accompanying drawings, in which:

FIGURE 1 (A and B) is a schematic representation
10 of a selection protocol of the invention, wherein Fig. 1A depicts a time line corresponding to the protocol depicted in Fig. 1B;

FIGURE 2 (A-C) are restriction maps of three
15 exemplary vectors for transfecting cells with the adenovirus E1A or VA1 genes;

FIGURE 3 (A-D) are restriction maps of exemplary
vectors carrying OP-1 as the reporter gene under
20 control of CMV-MIE constitutive "short" promoter;

FIGURE 4 is a restriction map of an exemplary
vector carrying the papilloma virus early region DNA;

25 FIGURE 5 is a restriction map of an exemplary
vector carrying a nucleotide sequence encoding a single
chain binding site as the reporter gene under control
of the CMV-MIE constitutive "short" promoter;

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DETAILED DESCRIPTION

A methodology and cell line useful for the large scale production of recombinant mammalian gene expression now has been discovered. The method has particular utility in providing useful quantities of protein encoded by "hard-to-express" genes. The method of the invention can produce stable, immortalized mammalian cell lines that constitutively express a gene of interest to produce a protein at a concentration of at least 1 μg protein/ 10^6 cells/ml without relying on high copy numbers of the gene of interest. Moreover, the method of the invention requires substantially shorter times for producing high expressing, fully amplified cells.

A general selection protocol useful in the method of the invention is depicted in Fig.1. As can be seen in step I of the protocol, an immortalized eukaryotic host cell, typically a mammalian cell, is transfected with nucleic acids carrying three DNA sequences. The first DNA sequence carries the reporter gene (gene of interest) operatively associated with a transcription promoter/enhancer unit of viral origin. The second DNA carries a viral gene encoding a transactivating protein competent to act on and stimulate transcription from the viral promoter unit that induces transcription of the reporter gene. The third DNA carries a viral gene encoding an RNA stabilizing sequence operative to promote translation of the reporter gene transcript. In Fig.1, these DNA sequences are carried on separate vectors and the vectors are transfected simultaneously. However, as will be appreciated by those having ordinary skill in the art, the DNA sequences can be transfected sequentially. For example, the cell first can be transfected with one or more nucleic acids carrying the

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translation promoter sequence and/or the transcription transactivator sequences, and stable integrants obtained, and the cell subsequently transfected with the reporter gene. In addition, two or three DNA
5 sequences can be carried on a single nucleic acid. Where two of the three sequences are carried on a single vector, combinations that may be particularly useful include the viral effector sequences, or the reporter gene and the translation effector sequence.
10 Where all three sequences are carried on a single vector, means for modulating expression of the transcription transactivator may need to be provided. For example, the adenovirus E1A gene generally is believed to interfere with cell growth at high levels
15 of expression. Where the E1A gene is transfected on a separate nucleic acid than that of the reporter genes, for example, the sequences can assort randomly, in a given cell, increasing the possibility of selecting for a clone that produces optimal protein levels of both
20 genes. Because the range of assortment events will be reduced where the genes occur on a single nucleic acid, means for modulating, e.g., limiting the E1A gene expression, for example, by means of a weakened promoter, may be required.

25 In step II of Figure 1, transfected cells are replated, e.g., in individual wells and in selective medium, grown to confluency, and the concentration of reporter protein produced (the protein of interest)
30 determined from aliquots of culture medium taken from each well, typically by ELISA or Western blot (step III.) Candidate cells then are cloned/amplified by multiple passages in a limited dilution series in the presence of increasing concentrations of amplification
35 medium, until high expressing, fully amplified cells are obtained, (step IV of Fig. 1.) Without being limited to a particular theory, transfection with the

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viral transcription transactivating gene appears to limit the degree of amplification allowed in a cell so maximum amplification clones can be achieved at a faster rate and, in the presence of lower
5 concentrations of toxic agent than in cells amplified in the absence of the viral effector genes. Moreover, the presence of the transcription and translation effector genes work synergistically to increase expression from each gene copy. In the method of the
10 invention, step IV occurs in fewer steps than in cells amplified in the absence of the viral effector genes, (typically requiring only about one month vs six months.) Once clones exhibiting the desired protein production level are obtained (step V), cloned cells
15 can be cultured in a large-scale production protocol (step VI) to produce large (at least 2 liters) quantities of the desired protein, which then can be purified from the culture medium using a standard, desired methodology (step VII).

20
Following the method outlined herein, stable, high producing clones can be obtained. The combination of viral effector genes has a synergistic effect on protein production, enhancing levels beyond those
25 achievable in the presence of only one of the two effector genes or by gene amplification alone for poorly expressed genes. Moreover, where the reporter gene is a poorly expressed gene capable of producing protein at less than 100-1000 ng/10⁶ cells/ml in a
30 highly amplified cell line, the method and cell line of the invention which combine the step of cotransfection with viral effectors and the step of amplification of the reporter gene unexpectedly can increase the level of protein produced beyond the level obtained using
35 either step alone.

Provided below are detailed descriptions of the various elements that comprise the methods and

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compositions of the invention, as well as methods for their application, and numerous, nonlimiting examples which 1) illustrate useful, exemplary vector constructions, transfection protocols, useful cell line
5 sources, and culturing, selection and subcloning protocols; 2) provide assays for testing candidate cell lines for their protein productivity and cell growth capabilities; and 3) provide methods for culturing cells in low serum or serum-free media. Also provided
10 are examples demonstrating the method of the invention with two genes known to be poorly expressed genes: a morphogenic protein representative of the class of tissue morphogens as defined herein (OP-1), and a gene encoding a single chain binding site (a non-native DNA
15 sequence).

I. Useful Cells

Any immortalized eukaryotic cell line suitable
20 for long term culturing conditions is contemplated to be useful in the method and compositions of the invention. Useful cell lines should be easy to transfect, are capable of stably maintaining foreign DNA with an unarranged sequence, and have the
25 necessary cellular components for efficient transcription, translation, post-translation modification, and secretion of the protein. Where the cell is to be transfected with a non-dominating selection gene, the cell genotype preferably is
30 deficient for the endogenous selection gene. Preferably, the cell line also has simple media composition requirements, rapid generation times, and can be adapted to grow in a suspension culture. Particularly useful cell lines are mammalian cell
35 lines, including myeloma, HeLa, fibroblast, embryonic and various tissue cell lines, e.g., kidney, liver, lung and the like. A large number of cell lines now

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are available through the American Type Culture Collection (ATCC, Rockville, Md) or through the European Collection of Animal Cell Cultures (ECACC) (Porton Down, Salisbury, SP4 0JG, U.K.).

5

Where the reporter gene encodes a morphogenic protein as defined herein, particularly useful cell lines are envisioned to include mammalian cell lines, including, without limitation, uro-genital cell lines, including kidney and bladder cells, lung, liver, cardiac muscle or other smooth muscle cell lines and other cell lines known to express endogenous genes encoding morphogenic proteins.

15 II. Useful Promoter Units for Reporter Genes

The reporter gene should be operatively associated with a promoter unit capable of being stimulated by a viral transacting transcription activator as described herein. Useful promoters include the human cytomegalovirus major intermediate-early promoter (hCMV-MIE) or the adenovirus early promoter (ElA, ElB promoter), or the adenovirus late region promoter. Preferably, the CMV-MIE promoter is an intron-free form of the promoter, so-called the CMV-MIE "short" promoter. CMV promoter sequences or plasmids containing them can be purchased commercially, e.g. from Invitrogen, Inc., San Diego (pCDM8) and from Clontech, Inc., Palo Alto. Preferably, the transcription further is stimulated by the inclusion of a cis-acting enhancer sequence, e.g., the mouse mammary tumor virus long terminal repeat (MMTV-LTR) or the Rous sarcoma virus long terminal repeat (RSV-LTR.) Enhancer sequences or plasmids containing them also are commercially available (e.g., from Invitrogen Inc., San Diego, or Clontech Inc., Palo Alto), and/or also are available through the ATCC and ECACC.

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III. Useful viral expression effector genes

5 The viral expression effector genes useful in the methods and cell lines of the invention are competent to act on the promoter that induces transcription of the reporter gene and/or to act on the reporter gene's transcript or the translation machinery.

10

At least one of the expression effector genes is a viral transacting transcription activator. Useful sequences include those encoded by the adenovirus-2 E1A and E1B genes, as well as by the bovine papilloma virus early region DNA. Details on these sequences and vectors carrying these sequences can be found in Maat, J. et al. (1979) Gene 6:75, and in EP 0378,382 and Cockett, (1990) Nucleic Acids Research 19: 319-325 all incorporated herein by reference. Whole bovine papilloma DNA virus can be obtained commercially, e.g., from IBI, New Haven (Catalog # 33040.)

25 The authors of EP 0378,382 state that appropriate levels of the transcription activator can be obtained by choice of a suitable promoter/enhancer unit for its transcription (e.g., a weak promoter is preferred and a stable transcription activator expressing cell is produced before transfection with the reporter gene.) Alternatively, and as currently preferred herein, the activator gene is co-transfected together with the reporter gene, and the transfected cells individually allowed to determine the appropriate, combined level of all recombinant, expressed genes, including the optimal level of the activator gene product for that cell when present in the cell in combination with the reporter gene and gene product.

30

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The second viral effector preferably is a translation activator, preferably an RNA sequence competent to enhance translation of the reporter gene.

5 Preferably, the RNA sequence is encoded by an adenovirus VA gene, preferably at least VA1. Other useful sequences include a portion of the bovine papilloma virus early region DNA. Details of these sequences also can be found in Maat, J., et al. (1979)

10 Gene 6:75, EPO 3378, 382 and Cockett et al. (1990) Nucleic Acids Research 19: 219-325; in Schneider et al. (1984) Cell 37:291 et. seq. and in Thimmappaya et al. (1982) Cell 31:543-551, the disclosures of which all are incorporated herein by reference. Like the

15 transcription activator sequence, the translation activator sequence may be transfected under control of its own promoter/enhancer unit, or under a stronger or weaker promoter unit. The choice of promoter/enhancer unit is less critical, as high expression clones having

20 the optimal combination of activator and reporter gene sequences will be determined empirically by the screening and selection protocol in the preferred embodiment of the invention.

25 By screening for good cell growth and selecting for high reporter gene expression, optimal concentrations of all elements for maximal expression of a given reporter gene more easily is obtained than by artificially predetermining the level any one

30 element should have in the cell.

IV. Vector Construction Considerations

Optimal vector design for transfection into

35 eukaryotic cells should include appropriate sequences to promote expression of the gene of interest as described supra, including appropriate transcription

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initiation, termination, and enhancer sequences, as well as sequences that enhance translation efficiency, such as the Kozak consensus sequence. Preferred DNA vectors also include a marker gene as a means for
5 selecting for the presence of the vector DNA in a cell. The marker gene also may provide means for amplifying the copy number of the gene of interest, and may also include a second gene for resistance to cytotoxins.

10 Substantial progress in the development of mammalian cell expression systems has been made in the last decade, and many aspects of these systems' features are well characterized. A detailed review of the state of the art of the production of foreign
15 proteins in mammalian cells, including useful cell lines, protein expression-promoting sequences, marker genes, and gene amplification methods, is disclosed in Bendig, Mary M., (1988) Genetic Engineering 7:91-127.

20 V. Transfection Considerations

Any method for incorporating nucleic acids into cells of interest is contemplated in the method of the invention. Calcium phosphate (CaPO_4), followed by
25 glycerol shock is a standard means used in the art for introducing vectors, particularly plasmid DNA into mammalian cells. A representative method is disclosed in Cockett et al. (1990) Biotechnology 8: 662-667, incorporated herein by reference. Other methods that
30 may be used include electroporation, protoplast fusion, particularly useful in myeloma transfections, microinjections, lipofections and DEAE-dextran mediated uptake. Methods for these procedures are described in F.M. Ausubel, ed., Current Protocols in Molecular John
35 Wiley & Sons, New York (1989).

- 30 -

Generally, plasmids are transfected in equal molar concentrations and cells are plated at a density of about $1-2 \times 10^6$ cells/dish. As will be appreciated by those having skill in the art, optimal DNA concentrations per transfection will vary by transfection protocol. For a calcium phosphate transfection, for example, preferably 5-10 μg plasmid DNA per plasmid type is transfected. So, where a simultaneous triple transfection is contemplated, 15-30 μg are transfected in total. In addition, the DNA to be transfected preferably is essentially free of contaminants that may interfere with DNA incorporation. A standard means used in the art for purifying DNA is by ethidium bromide banding.

15

VI. Amplification Considerations

One of the better characterized methods of gene amplification in mammalian cell systems is the use of the selectable DHFR gene in a dhfr- cell line. Generally, the DHFR gene is provided on the vector carrying the gene of interest, and addition of increasing concentrations of the cytotoxic drug methotrexate leads to amplification of the DHFR gene copy number, as well as that of the associated gene of interest. DHFR as a selectable, amplifiable marker gene in transfected Chinese hamster ovary cell lines (CHO cells) is particularly well characterized in the art. The instant invention may be practiced using this particular amplification marker. Other useful amplifiable marker genes include the adenosine deaminase (ADA) and glutamine synthetase (GS) genes. (See, R.E. Kellems, Gene Amplification in Mammalian Cells, Marcel Decker (1992).

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VII. Exemplary Large Scale Culture Production
Protocols

Any means available or known in the art for
5 large scale eukaryotic cell culturing (e.g., at least 2
liters) is anticipated to be useful for culturing the
cell lines taught by this invention. Two general
culturing methodologies practiced in the art are the
"continuous flow" systems, where cells are exposed to
10 fresh media at regular intervals to replenish any spent
nutrients, and the "terminal" or batch culture system,
where cells are grown to confluency under a defined set
of culture parameters, and the production medium
harvested when cells have entered post-logarithmic
15 phase. In addition, cells may be grown as a suspension
culture or as attached, monolayers of cells.

The type of culture system used and the media
replenishment regimen chosen are determined by the host
20 cell line requirements. For example, some mammalian
cell lines are not adaptable to suspension cultures
while others are unable to remain securely attached to
a substrate. Additionally, some cell lines are highly
vulnerable to the shear forces associated with
25 suspension and/or bioreactor culture conditions. In the
case of these cell lines, addition of agents such as
anti-foam and/or shear-minimizing agents may permit use
of suspension cultures. Another factor critical in the
choice of culture system is the host cell line's gas
30 requirements, with gas transfer and gas composition
being two important considerations for optimal cell
growth in vitro. Numerous references are available that
describe means for creating large scale culture
conditions and general considerations. Exemplary
35 references include R.J. Freshney, Animal Cell Culture:
A Practical Approach 2d.ed., Oxford University Press,
New York, 1992, and M. Butler, Mammalian Cell

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Biotechnology: A Practical Approach, Oxford University Press, New York, 1991.

VIII. Media Considerations

- 5 Transfectants obtained with the above-described preferred protocol are initially conditioned in media containing serum proteins. Preferably, under production conditions, the cells are adapted to growth
- 10 in low serum or serum-free conditions, to limit interference with protein purification. Useful media includes media containing 0.1%-0.5% dialyzed fetal calf serum. In a preferred embodiment, the low serum or serum-free media is supplemented with one or more lipid
- 15 membrane phosphoglyceride ester degradation products, as disclosed in USSN 08/124,676, filed September 22, 1993 and incorporated herein by reference. Other media components useful in production protocols include protease inhibitors. A representative reference
- 20 detailing growth supporting media considerations for mammalian cell culture includes ATCC Media Handbook, Cote et al., ed., American Type Culture Collection, Rockville, MD (1984).
- 25 As indicated above and as will be appreciated by those having ordinary skill in the art, particular details of the conventional means for transfection, expression, and purification of recombinant proteins are well documented in the art and are understood by
- 30 those having ordinary skill in the art. The instant invention enables and discloses improvements to these conventional means comprising a combination of transfection vectors which achieves markedly enhanced recombinant expression of low expressing genes
- 35 including genes encoding morphogenic proteins, using immortalized, eukaryotic cells.

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Further details on the various technical aspects of each of the steps used in recombinant production of foreign genes in mammalian cell expression systems can
5 be found in a number of texts and laboratory manuals in the art, such as, for example, F.M. Ausubel et al., ed., Current Protocols in Molecular Biology, John Wiley & Sons, New York, (1989.)

10 Example 1. CONSTRUCTION OF REPRESENTATIVE
EXPRESSION EFFECTOR VECTORS

The pH1176 vector (FIG 2A) employs the adenovirus E1A gene (Seq. ID No. 1) under the control of the
15 thymidine kinase promoter as a transactivating transcription activator. The E1A coding region was isolated by polymerase chain reaction (PCR) with adenovirus DNA as template and priming the reaction with synthetic oligo nucleotide primers to gene
20 terminal sequences on the upper and lower strands (Seq. ID Nos. 2 and 3) with a commercially available thermal cycler and Reagent kit (e.g., GeneAmp, Perkins-Elmer Corp., Norwalk) and following manufacturer's instructions in a standard protocol. (See, for
25 example, Saiki et al. (1985) Science 230:1350-1354.) The fragment was cloned in a standard, commercially available pUC cloning vector e.g., SK-Bluescript, Stratagene, Inc., Palo Alto. The herpes simplex virus thymidine kinase promoter was isolated as a 5' SalI to
30 3' HindII fragment from another plasmid (pTK-HGH, Allegro Systems, San Juan Capistrano) and fused to a StuI site upstream of the E1A coding region. The fragment bearing the TK-promoter and E1A was cloned in a pUC plasmid resulting in plasmid pH1176 (Fig. 2A).
35 Seq. ID No. 1 describes the nucleotide sequence of the Pst 1 - ECORI fragment of pH 1176. Maintenance of low copy number in the transfected cells under selective

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pressure can be verified to ensure incorporation and maintenance of the ElA gene, e.g., by Southern blot or gel assay wherein the level of detected target DNA is compared to a known quantity.

5

The pH989 (FIG 2B) and pH1130 (FIG 2C) vectors employ the adenovirus VA1 gene (Seq. ID No. 4) as a translation stimulator (e.g., an RNA sequence competent to promote translation of the transcript encoded by the gene of interest. Cloning of the adenovirus VA1/VA2 gene complex was achieved as follows: the VA1 and VA2 complex was isolated from adenovirus DNA by PCR using two synthetic oligo-nucleotides to gene terminal sequences (upper and lower strands)(Seq. ID Nos. 5 and 6) and standard conditions for PCR. The primers added a new upstream PstI site and a downstream PvuII site. The PstI to PvuII fragment was cloned into the PstI and EcoRV sites of the SK(-) Bluescript cloning vector (Stratagene, Inc., Palo Alto, CA) resulting in plasmid pJ13: plasmid pJ13 was then incorporated into final vectors pH 989 and pH 1130 as follows.

In the case of pH989, the neo expression element was from the pMamneo expression vector (Clontech, Inc.) was subcloned into the BamHI site of a standard pUC cloning vector, resulting in plasmid pH989. The plasmid orientation (with regard to the BamHI insert) in which the neo gene is colinear with the lac promoter of pUC was chosen for the next step, the addition of the VA1 gene. The VA1 DNA was excised from pJ13 at flanking poly-linker sites, SpeI and ClaI, and the fragment was inserted between the XbaI and ClaI sites of the plasmid pH988 resulting in pH989.

In the case of pH1130, a modified dhfr gene containing a minimal upstream untranslated region was constructed by site directed mutagenesis and a 5' PvuII

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site was introduced only a few nucleotides upstream of the ATG initiation condon. At the 3' end a SalI site was added next to a natural BglII site. Seq. ID No. 4 describes the nucleotide sequence of the Pst 12 - EcoRI
5 fragment of the pH1130 vector and includes both the VA gene sequence (e.g., nucleotides 1 to 1330) and the DHFR gene sequence.

The tailored dhfr gene was then inserted into
10 plasmid pH989 in place of the neo gene. For this purpose pH989 was opened at a unique StuI site, located between the SV40 promoter and the neo gene, and at a unique SalI site, downstream or 3' of the neo gene and dhfr, as a PvuII to SalI fragment, was inserted. The
15 end of PvuII and StuI sites are compatible for ligation and both sites are lost in the process. The resulting plasmid, pH1130, contains VA1 and dhfr.

Example 2. MORPHOGEN DNA VECTORS

20

FIG. 3 (A-D) discloses restriction maps of various exemplary expression vectors designed for OP1 expression in mammalian cells. Each of these vector constructs employs a full-length hOP1 cDNA sequence
25 originally isolated from a human cDNA library (human OP1 see Ozkaynak et al. (1990) EMBO, incorporated herein above by reference, and subsequently cloned into a conventional pUC vector (pUC-18) using pUC polylinker sequences at the insertion sites. The hOP1 cDNA
30 fragment cloned into each of these constructs is either the intact SmaI-BamHI hOP1 cDNA fragment (nucleotides 26-1385 of Seq. ID No. 1 as disclosed in U. S. S. N. 841, 646 filed February 21, 1992, soon to issue as US Pat.No. _____, or Ozkaynak, (1990) EMBO
35 J. 9:2085-2093 incorporated herein above by reference) or modifications of this fragment where the flanking

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non-coding 5' and/or 3' sequences have been trimmed back, using standard molecular biology methodology. Each vector also employs an SV40 origin of replication (ori). In addition, the early SV40 promoter is used to drive transcription of marker genes on the vector (e.g., neo and DHFR). It will be appreciated by those skilled in the art that DNA sequences encoding truncated forms of morphogenic protein also may be used, provided that the expression vector or host cell then provides the sequences necessary to direct processing and secretion of the expressed protein.

The pH717 expression vector (FIG 3A) contains the neomycin (neo) gene as a selection marker. This marker gene is well characterized in the art and is available commercially. Alternatively, other selectable markers may be used. The particular vector used to provide the neo gene DNA fragment for pH717 may be obtained from Clontech, Inc., Palo Alto, CA (pMAM-neo-blue). This vector also may be used as the backbone. In pH717, hOPl DNA transcription is driven by the CMV promoter, boosted by the RSV-LTR and MMTV-LTR (mouse mammary tumor virus) enhancer sequences. These sequences are known in the art, and are available commercially. For example, vectors containing the CMV promoter sequence may be obtained from Invitrogen Inc., San Diego, CA, (e.g., pCDM8).

The pH752 and pH754 expression vectors contain the DHFR gene, under SV40 early promoter control, as both a selection marker and as an inducible gene amplifier. The DNA sequence for DHFR is well characterized in the art, and is available commercially. For example, pH754 may be generated from pMAM-neo (Clontech, Inc., Palo Alto, CA) by replacing the neo gene (BamHI digest) with

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an SphI-BamHI, or a PvuII-BamHI fragment from pSV5-DHFR (ATCC #37148), which contains the DHFR gene under SV40 early promoter control. A BamHI site can be engineered at the SphI or PvuII site using standard techniques (e.g., by linker insertion or site-directed mutagenesis) to allow insertion of the fragment into the vector backbone. hOP1 DNA can be inserted into the polylinker site downstream of the MMTV-LTR sequence (mouse mammary tumor virus LTR), yielding pH752 (FIG. 3B). The CMV promoter sequence then may be inserted into pH752 (e.g., from pCDM8, Invitrogen, Inc.), yielding pH754 (FIG. 3C.) The SV40 early promoter, which drives DHFR expression, is modified in these vectors to reduce the level of DHFR mRNA produced. Specifically, the enhancer sequences and part of the promoter sequence have been deleted, leaving only about 200 bases of the promoter sequence upstream of the DHFR gene.

The pW24 vector (FIG 3D), is essentially identical in sequence to p754, except that neo is used as the marker gene (see pH717), in place of DHFR.

The pW24 plasmid contains OP-1 cDNA under transcriptional control of the CMV (cytomegalovirus) immediate early promoter. This promoter is derived from pCDM8 and is much shorter than the CMV promoter used by other practitioners. The latter one contains introns and also additional upstream sequences. The selective marker on pW24 is the neo gene which supplies resistance to the cytostatic drug G418. In addition the plasmid contains the LTR from rous sarcoma virus and from mouse mammary tumor virus and the SV40 origin of replication. The latter viral enhancer elements (LTR and SV40 ori) are not essential even though they may have some beneficial effects on expression.

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Plasmid pW24 was constructed by a 3-part assembly essentially as follows: The pMam-neo vector (Clontech Inc., Palo Alto, CA) was opened in its
5 poly-linker/cloning site by restriction digest with NheI and SalI. The CMV promoter from pCDM8 and the human OP-1 gene was then inserted on a SpeI to SalI fragment. During this ligation the NheI and SpeI sites which have compatible ends were both lost.

10

Prior to this, the CMV promoter had been joined with the OP-1 cDNA, by fusion of the 3' XbaI site flanking the CMV promoter with a 5' NheI site placed upstream of the OP-1 cDNA, in an earlier step. During
15 this ligation the XbaI site and NheI site which have compatible ends were also both lost. The NheI site, flanking OP-1 cDNA was acquired previously when OP-1 cDNA, on a fragment spanning from its natural 5' SmaI site, approximately 20 nucleotides upstream of the ATG,
20 up to the natural BamHI site, approximately 40 nucleotides downstream of the stop codon, with a SalI site attached immediately next to it and was inserted into the pMAMneo vector between the SmaI and SalI sites of its poly-linker insertion site.

25

Example 3. TRANSFECTIONS

In all examples, transfections were by calcium phosphate coprecipitation, performed using standard
30 procedures. CHO cells, from Dr. Lawrence Chasin, Columbia University, NY, were cultured in α MEM, containing 5% or 10% fetal bovine serum (FBS), non-essential amino acids, glutamine and antibiotics: penicillin and streptomycin, all being obtained from
35 GIBCO, New York. CHO cells transfected with vectors containing a neo gene were cultured in the same growth medium containing the toxin G418 (0.4 mg/ml). CHO

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cells transfected with vectors containing the DHFR selectable amplifiable gene, were cultured in α -MEM (α MEM lacking thymidine, glycine and hypoxanthine), 10% dialyzed FBS, and methotrexate (MTX) at 0.02-0.1 μ M.

5

Stable cell line transfections were carried out by seeding $1-2 \times 10^6$ cells in a 9 cm. petri dish. Following up to 24-hour incubation in growth medium, each petri dish was transfected with 10-30 μ g total vector DNA in equimolar amounts, by calcium phosphate coprecipitation followed by glycerol shock using standard methodology. Cells are incubated at 37°C in growth medium for 24 hours, then transferred to selection medium. All cultures were fed once or twice weekly with fresh selective medium. After 10 - 21 days, resistant colonies were picked and assayed for protein production.

Table I summarizes the actual experimental transfections performed to determine the effect of the above-described vectors (alone and in combination) on OP1 production. The vectors and the combinations presented in Table I were selected, in part, to determine the optimal configuration of OP1, VA1, and ElA vectors, and in part to determine whether the ElA and/or VA1 genes are crucial for optimal OP1 expression. In Table I, transfections number 1, 2 and 3 are considered "double" transfections since two different genes (not necessarily vectors) have been introduced to the CHO host cell; similarly, transfections number 4 and 5 are considered "triple" transfections since three different genes (not necessarily vectors) have been introduced. "Single" transfections refer to CHO cells transfected only with an OP1-encoding vector.

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TABLE I

TRANSFECTION	PLASMID (element present/selection marker)
5	
1	pH754 (OP-1/DHFR) + pH989(VA1/neo)
2	pW24 (OP-1/neo) + pH1130(VA1/DHFR)
3	pH754 (OP-1/DHFR) + pH1176(E1A/none)
4	pW24 (OP-1/neo) + pH1130(VA1/DHFR) + pH1176(E1A/none)
10	5 pH754 (OP-1/DHFR) + pH989(VA1/neo) + pH1176(E1A/none)

Example 4. SELECTION SCREENING FOR
CANDIDATE CELL LINES

15 Following transfection and growth in selection medium, cells were screened for candidates to be subcloned, essentially as described in Figure 1.

20 Using the transfection schemes summarized in Table I above, approximately 30 individual clones from each transfection in Table I were selected, transferred to a 24-well petri dish, and allowed to grow to confluence in serum-containing media. The conditioned media from all surviving clones was screened for protein
25 production using a standard ELISA (enzyme-linked immunosorbent assay) or Western blot. The methodologies for these assay protocols as well as for generating antibodies for use in these assays are well described in the art (see, e.g., Ausubel, and USSN
30 08/040,510, filed March 31, 1993 and incorporated herein by reference). A summary of this primary ELISA screening data for OP1 is found in Table II.

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TABLE II

OP-1 PRODUCTION BY CHO TRANSFECTANTS

RELATIVE PROTEIN PRODUCTION	TRANSFECTION NUMBER (from TABLE I) (number clones/category)				
	1(VA1)	2(VA1)	3(E1A)	4(VA1, E1A)	5(VA1, E1A)
-	24	24	13	8	13
±	1	4	1	5	0
+	2	2	0	3	6
++	0	0	0	9	8
+++	0	0	1	5	9
Total Screened:	27	30	15	30	36

The data in Table II suggest that the VA1 and E1A genes act synergistically to enhance OP1 expression in unamplified transfected CHO cells. Transfectants resulting from transfection number 1, 2, or 3 produce negligible amounts of OP1, while transfection number 4 and 5 resulted in approximately 57% and 64%, respectively, of the transfectants producing elevated levels of OP1.

Example 5. AMPLIFICATION SUB-CLONING / CLONING METHOD

Candidate cell lines identified by the screening protocol of Example 4, then were seeded on ten 100mm petri dishes at a cell density of either 50 or 100 cells per plate, and with a higher MTX concentration (e.g., 1.0-5 μ m).

After 10-21 days of growth, clones are isolated using cloning cylinders and standard procedures, and cultured in 24-well plates. Clones then are screened for OP1 expression by Western immunoblots using

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standard procedures, and OP1 expression levels compared to parental lines. Candidate cells showing higher protein production than cells of parental lines then are replated and grown in the presence of a still
5 higher MTX concentration (e.g., 5-20 μ m). Generally, no more than 2-3 rounds of these "amplification" cloning steps are necessary to achieve cell lines with high protein productivity. Useful high producing cell lines may be further subcloned to improve cell line
10 homogeneity and product stability.

Example 6. CHARACTERIZATION OF TRANSFECTED CLONE

a) Copy Number

15 Southern blots, using standard methodology, may be used to assess the state of integrated sequences and the extent of their copy number amplification in the host genome. Copy number experiments on various
20 transfections indicates that the triple transfectants, fully amplified, have on the order of 10 copies of the reporter gene, double transfectants of the reporter gene with VA1 have a somewhat higher copy number, (on the order of 100 copies/cell), both of which are
25 significantly smaller than the copy number for single transfectants, fully amplified. (See Table III, below).

b) mRNA Measurements

30 Transcription levels of transfected OP1 sequences can be measured in the different expression systems by analyzing mRNA levels (Northern blots), using total cellular RNA and conventional hybridization
35 methodology. Northern blots on various transfections indicate that OP1 transcript production is enhanced in double transfectants of OP1/VA1 as compared with single

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transfectants, more enhanced in double transfectants of OP1/E1A, and still more enhanced in triple transfectants.

5 c) Protein Measurements

Protein levels may be measured by Western blots (immunoblots) using rabbit antisera against the protein product of interest. Western blot methodologies are
 10 will known to those skilled in the art, and may be performed using commercially-available reagents, USSN 08/040,510, incorporated herein above by reference, describes methods for obtaining OP1 antisera and antibodies, as well as various immunoassays. The
 15 protein data presented in Table III is for a "terminal" or "batch" culture where protein is harvested when cells have reached post-logarithmic phase.

Interestingly, protein production in the triple
 20 transfectant is synergistically enhanced as compared with either single or double transfectants, even though the copy number is significantly lower in these cells and transcript levels are only moderately enhanced.

25

Table III

OP1 PRODUCTION BY CHO TRANSFECTANTS

30	TRANSFECTION		OP1($\mu\text{g}/10^6$ cell/ml)	Copy Number/Cell
	CATEGORY	PLASMID		
35	Single	pH754(OP1)	≤ 1	1000
	Double	pW24/pH1130(OP1/VA1)	$\leq 1-2$	100
	Triple	pW24/pH1176/pH1130(OP1/E1A/VA1)	$\geq 5-10$	10

40

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These data demonstrate that the invention's combination of transfecting DNA sequences markedly enhances production of OP1. As set forth in Table III, triple transfectants produce levels of OP1 which are substantially greater than those produced by single transfectants using significantly fewer copies of the gene, while both categories of double transfectants produce approximately intermediate levels of the OP1 produced by triple transfectants. On the basis of these data, it appears that the transcription activator E1A and the translation enhancer VA1 act synergistically resulting in high expression of the OP1 gene.

Example 7. PROTEIN PRODUCTION IN LARGE SCALE PROTOCOLS

A currently preferred method of large scale protein production e.g., at least 2 liters, is by suspension culturing of the host CHO cells. Chinese hamster ovary cells prefer attachment but can be adapted to grow in suspension mode of cultivation. The cells were trypsinized from the dish, introduced to growth media containing 10% FBS and using a pipet, completely suspended to ideally achieve a single cell suspension. This was introduced to a spinner flask and placed in a 37°C 95% air/5% CO₂ humidified incubator. Over a period of time the cells were subcultured in medium with descending concentrations of serum. Within the spinner flasks there is a balance between sufficient agitation rate to maintain a single cell suspension and the shear force associated with the agitation impeller. Due to the nature of 95%air/5% CO₂ incubators there is a balance between oxygen absorption/CO₂ desorbtion in the medium and agitation rate, as well as, surface to volume ratios.

- 45 -

For example, the production of CHO conditioned medium in suspension cultures was carried out as follows: The adapted cells were introduced into a 3L spinner flask at an initial viable cell density of approximately 2×10^5 cells/ml. The culture medium was DMEM/F-12 (1:1) (GIBCO, New York) supplemented with 2% FBS. The agitation was approximately 50-60 rpm with a paddle impeller. The culture volume was 1500mls (half max.) in order to increase relative surface to volume ratio. After 7 days the culture media is harvested, centrifuged at 1500 rpm and the clarified conditioned media stored at 4°.

Example 8. CHARACTERIZATION OF OP1 EXPRESSED BY
PREFERRED CLONES

Standard biochemical procedures, including apparent molecular weight determined by gel electrophoresis, and/or N-terminal and C-terminal sequencing (by CNBr analysis) can be used to verify the form and structure of the protein produced. Using these methodologies (described in numerous texts available in the art and, for example, in USSN 841,646) the OP1 protein was found to be of the same form as in cells of single transfectants, e.g., mature, full length form (139 amino acids) as well as various N terminally truncated forms, which are characteristic of expression in CHO cells. (See USSN 841,646).

Moreover, using a standard bioassay methodology for verifying the biological activity of osteogenic (morphogenic proteins), e.g., rat ectopic bone formation assay (see US 5,011,691) the biological activity of the protein produced by the method of the invention was determined to be substantially the same half maximum specific activity as that of the protein produced by single transfectants (see, for example,

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USSN 07/841,646.) Moreover, in all cases successful implants exhibit a controlled progression through the states of matrix-induced endochondral bone development including: transient infiltration by polymorphonuclear leukocytes on day one; mesenchymal cell migration and proliferation on days two and three; chondrocyte appearance on days five and six; cartilage matrix formation on day seven; cartilage calcification on day eight; vascular invasion, appearance of osteoblasts, and formation of new bone on days nine and ten; appearance of osteoblastic and bone remodeling and dissolution of the implanted matrix on days twelve to eighteen; and, hematopoietic bone marrow differentiation in the ossicle on day twenty-one.

15

Example 9. GENERAL ISOLATION/PURIFICATION SCHEME
 FOR MORPHOGENS

A representative purification scheme for purifying the recombinant morphogenic proteins that is rapid and highly effective is described in USSN 841,646 and is provided below. The protocol described below involves three chromatographic steps (S-Sepharose, phenyl-Sepharose and C-18 HPLC), and produces OP1 of about 90% purity. The purification protocol of choice will vary with the gene to be expressed.

An alternative protocol that also produces protein of high purity is a variant three step chromatography protocol utilizing Zn/IMAC (metal affinity chelating chromatography), hydrophobic interaction chromatography (e.g., phenyl-Toyopearl) and reverse phase (C-18) chromatography. Still other useful related chromatography methods include heparin-Sepharose used in combination with the S-Sepharose column.

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For a typical 2L preparation of transfected CHO cells conditioned in 0.5% FCS, the amount of OP1 in the media, estimated by Western blot, is about 10-20mg/L.

Briefly, OP1-containing culture media is diluted to
5 6M urea, 0.05M NaCl, 13mM HEPES, pH 7.0 and loaded onto an S-Sepharose column, which acts as a strong cation exchanger. OP1 binds to the column in low salt, and serum proteins are removed. The column is subsequently developed with two step salt elutions. The first
10 elution (0.1M NaCl) removes contaminants and approximately 10% of the bound OP1. The remaining 90% of OP1 then is eluted in 6M urea, 0.3M NaCl, 20mM HEPES, pH 7.0.

15 Ammonium sulfate is added to the 0.3M NaCl fraction to obtain final solution conditions of 6M urea, 1M $(\text{NH}_4)_2\text{SO}_4$, 0.3M NaCl, 20mM HEPES, pH 7.0. The sample then is loaded onto a phenyl-Sepharose column (hydrophobic interaction chromatography). OP1 binds
20 phenyl-Sepharose in the presence of high concentrations of a weak chaotropic salt (e.g., 1M $(\text{NH}_4)_2\text{SO}_4$). Once OP1 is bound, the column is developed with two step elutions using decreasing concentrations of ammonium sulfate. The first elution (containing 0.6M $(\text{NH}_4)_2\text{SO}_4$)
25 primarily removes contaminants. The bound OP1 then is eluted with a 6M urea, 0.3M NaCl, 20mM HEPES, pH 7.0 buffer containing no ammonium sulfate.

The OP1 eluted from the phenyl-Sepharose column is
30 dialyzed against water, followed by 30% acetonitrile (0.1% TFA), and then applied to a C-18 reverse phase HPLC column. Gel separation of oxidized and reduced OP1 samples show that the reduced and oxidized subunits appear to be identical to that of the naturally-sourced
35 OP purified from bone.

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An alternative chromatography protocol (also disclosed in USSN 07/841,646 filed February 21, 1992, soon to issue as U.S. Patent No. _____, and
5 herein incorporated by reference) is to perform the S-Sepharose chromatography in the absence of 6 M urea. The bound proteins then are eluted with salt step elutions (e.g., 100-400 mM NaCl). Most of the OP1 is eluted with about 300 mM NaCl. Additional OP1 then can
10 be eluted with 300 mM NaCl in the presence of 6M urea. The 6M urea elution also may be used in place of the non-urea elution to achieve maximum recovery in one step. In addition, OP1 may be eluted from the phenyl-Sepharose column in 38% ethanol-0.01% TFA, thereby
15 eliminating the need to dialyze the eluent before applying it to the C-18 column. Finally, multiple C-18 columns may be used (e.g., three), to further enhance purification and concentration of the protein.

20 OP1 also will bind hydroxyapatite efficiently, but only in the absence of 6 M urea and at low phosphate concentrations (less than 5 mM phosphate). Bound OP1 can be removed from the column with a step elution of 1 mM to 0.5M phosphate (in 0.5 M NaCl, 50 mM Tris, pH
25 7.0). OP1 elutes at about 250 mM phosphate. Additionally, urea (6M) may be added during the elution step.

Example 10. BPV EARLY REGION DNA CO-TRANSFECTION

30

Using the transfection and screening protocols described in Fig.1 and Examples 3 and 4, the effect of bovine papilloma virus early DNA on recombinant OP-1 DNA expression was tested. Here, a vector carrying BPV
35 early region DNA (BPV-1/pML2d vector, Fig. 4, purchased from IBI, New Haven) was transfected into a stable,

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unamplified OP-1 producing cell line, where the OP-1 gene was present at low copy number (on the order of 1-10 copies.) Alternatively, an E1A-producing vector (e.g., pH1176) was transfected into the same OP1-producing cell line. Candidates screened after the initial transfection demonstrated a 5-8 fold increase in the "double" transfectant (BPV/OP1), over the level of protein produced by the cell alone (OP1) and a 10 fold increase in the alternate "double" transfectant (E1A/OP1), as compared to the cell alone (OP1). Still higher comparative protein levels are anticipated to be detected when the candidate cells are subjected to amplification, cloning and subcloning protocols as described in Examples 5 and 6, to produce clones capable of producing the reporter gene at levels of at least 1, and preferably at least 5 μg protein/ 10^6 cells/ml, where protein is harvested from the medium in a batch culture when cells are in post-logarithmic phase. It is also anticipated that triple transfectants will further enhance protein production. A possible advantage of using BPV over a transactivating sequence like E1A, is in possible secondary effects on growth rates or media requirements conferred on the host cell.

25

Example 11. Recombinant Single Chain Fv

Using the protocols described in Fig. 1 and Examples 3-5, the method and constructs of the invention were tested on two different artificial genetic sequences encoding single chain Fvs, ("sFv"). These proteins also are referred to in the art as biosynthetic antibody binding site molecules ("BABS"). The two constructs, 741F8 and MOPC315, are well described in the art and in U.S. patent application (Atty. Docket No. CRP-093 filed on even date herewith,

35

- 50 -

the disclosure of which is incorporated herein by reference. The 741F8 construct interacts specifically with the c-erb2 antigen, a known marker associated with breast cancer. MOPC315 recognizes dinitrophenol. An
5 exemplary plasmid, pH1512, carrying the 741F8 sequence under control of the CMV-MIE "short" promoter, and containing a secretion signal sequence obtained from the heavy chain of the 520C9 monoclonal antibody DNA
10 sequence, is presented in Fig. 5. The MOPC315 DNA sequence used also was under control of the CMV-MIE promoter and had its own (native) secretion signal sequence.

Following the protocols described in Examples 3-5,
15 triple transfections of CHO cells were performed with an ElA-containing plasmid (e.g., pH1176), a VA1-containing plasmid (pH1130), and either the MOPC315 or the 741F8 vector. In all cases, high production clones produced at least between 1-6 μ g protein per
20 10^6 cells/ml in batch cultures, where protein was harvested from the medium when cells were in post-logarithmic phase.

The invention may be embodied in other specific
25 forms without departing from the spirit or essential characteristics thereof. The present embodiments are therefore to be considered in all respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather
30 than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (A) APPLICANT: CREATIVE BIOMOLECULES, INC.
(B) STREET: 45 SOUTH STREET
(C) CITY: HOPKINTON
(D) STATE: MA
10 (E) COUNTRY: USA
(F) POSTAL CODE (ZIP): 01748
(G) TELEPHONE: 508-435-9001
(H) TELEFAX: 508-435-0454
15 (I) TELEX:
- (ii) TITLE OF INVENTION: METHODS AND COMPOSITIONS FOR HIGH PROTEIN
PRODUCTION FROM RECOMBINANT DNA
- (iii) NUMBER OF SEQUENCES: 6
- 20 (iv) CORRESPONDENCE ADDRESS
(A) ADDRESSEE: PATENT ADMINISTRATOR, CREATIVE BIOMOLECULES, INC
(B) STREET: 45 SOUTH STREET
(C) CITY: HOPKINTON
25 (D) STATE: MA
(E) COUNTRY: USA
(F) ZIP: 07148
- (v) COMPUTER READABLE FORM:
30 (A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- 35 (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:
- 40 (viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: KELLEY, ROBIN D.
(B) REGISTRATION NUMBER: 34,637
(C) REFERENCE/DOCKET NUMBER: CRP-084PC
- 45 (ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 617/248-7000
(B) TELEFAX: 617/248-7100

50 (2) INFORMATION FOR SEQ ID NO: 1:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1356 base pairs
(B) TYPE: nucleic acid
55 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

5 (A) NAME/KEY: misc_feature
(B) 1..1356
(C) OTHER INFORMATION: /note= "adeE1A"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

10 CTGCAGGTCC TGCTTCATCC CCGTGGCCCG TTGCTCGCGT TTGCTGGCGG TGTCCCCGGA 60
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15 ATTGGCGAAT TCGAACACGC AGATGCAGTC GGGGCGGCGC GGTCCCAGGT CCACTTCGCA 180
TATTAAGGTG ACGCGTGTGG CCTCGAACAC CGAGCGACCC TGCAGCGACC CGCTTAACAG 240
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20 AGTCTTTTGG ACCAGCTGAT CGAAGAGGTA CTGGCTGATA ATCTTCCACC TCCTAGCCAT 360
TTTGAACCAC CTACCCTTCA CGAACTGTAT GATTTAGACG TGACGGCCCC CGAAGATCCC 420
25 AACGAGGAGG CGGTTTCGCA GATTTTCCC GAGTCTGTAA TGTGGCGGT GCAGGAAGGG 480
ATTGACTTAT TCACTTTTCC GCCGGCGCCC GGTCTCCGG AGCCGCCTCA CCTTTCCCGG 540
CAGCCCGAGC AGCCGGAGCA GAGAGCCTTG GGTCCGGTTT CTATGCCAAA CCTTGTGCCG 600
30 GAGGTGATCG ATCTTACCTG CCACGAGGCT GGCTTTCCAC CCAGTGACGA CGAGGATGAA 660
GAGGGTGAGG AGTTTGTGTT AGATTATGTG GAGCACCCCG GGCACGGTTG CAGGTCTTGT 720
35 CATTATCACC GGAGGAATAC GGGGGACCCA GATATTATGT GTTCGCTTTG CTATATGAGG 780
ACCTGTGGCA TGTTTGTCTA CAGTAAGTGA AAATTATGGG CAGTCGGTGA TAGAGTGGTG 840
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40 TGTGATTTTT TAAAAGGTCC TGTGTCTGAA CCTGAGCCTG AGCCCGAGCC AGAACCGGAG 960
CCTGCAAGAC CTACCCGGCG TCCTAAATTG GTGCCTGCTA TCCTGAGACG CCCGACATCA 1020
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CCTCCTGAGA TACACCCGGT GGTCCCCTG TGCCCCATTA AACCAGTTGC CGTGAGAGTT 1140
GGTGGGCGTC GCCAGGCTGT GGAATGTATC GAGGACTTGC TTAACGAGTC TGGGCAACCT 1200
50 TTGGACTTGA GCTGTAAACG CCCCAGGCCA TAAGGTGTAA ACCTGTGATT GCGTGTGTGG 1260
TTAACGCCTT TGTTTGTCTA ATGAGTTGAT GTAAGTTTAA TAAAGGGTGA GATAATGTTT 1320
55 AACTTGCATG GCGTGTTAAA TGGGGCGGGG AGATCT 1356

- 53 -

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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(ix) FEATURE:

- (A) NAME/KEY: misc feature
(B) LOCATION: 1..36
15 (C) OTHER INFORMATION: /note= "E1Aprim1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

20 AAAGGCCTCC ATGAGACATA TTATCTGCCA CGGAGG

36

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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(ix) FEATURE:

- (A) NAME/KEY: misc feature
(B) LOCATION: 1..28
35 (C) OTHER INFORMATION: /note= "E1Aprim2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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28

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- 45 (A) LENGTH: 2037 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

- 55 (A) NAME/KEY: misc feature
(B) LOCATION: 1..2037
(C) OTHER INFORMATION: /note= "adeVA1"

- 54 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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5	CTCGCCCTCC TCCAAGTCCA GGTAGTGCCG GGCCCGGCGC CGCGGGGGTT CGTAATCACC	120
	ATCTGCCGCC GCGTCAGCCG CGGATGTTGC CCCTCCTGAC GCGGTAGGAG AAGGGGAGGG	180
10	TGCCCTGCAT GTCTGCCGCT GCTCTTGCTC TTGCCGCTGC TGAGGAGGGG GGCGCATCTG	240
	CCGCAGCACC GGATGCATCT GGGAAAAGCA AAAAAGGGGC TCGTCCCTGT TTCCGGAGGA	300
	ATTTGCAAGC GGGGTCTTGC ATGACGGGGA GGCAAAACCC CGTTGCGCCG AGTCCGGCCG	360
15	GCCCCGAGACT CGAACCGGGG GTCCTGCGAC TCAACCCTTG GAAAATAACC CTCCGGCTAC	420
	AGGGAGCGAG CCACTTAATG CTTTCGCTTT CCAGCCTAAC CGCTTACGCC GCGCGCGGCC	480
20	AGTGGCCAAA AAAGCTAGCG CAGCAGCCGC CGCGCCTGGA AGGAAGCCAA AAGGAGCGCT	540
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	CGGCGGACGG CCGGATCCGG GGTTCGAACC CCGGTGCTCC GCCATGATAC CCTTGCGAAT	660
25	TTATCCACCA GACCACGGAA GAGTGCCCGC TTACAGGCTC TCCTTTTGCA CGGTCTAGAG	720
	CGTCAACGAC TGCGCACGCC TCACCGGCCA GAGCGTCCCG ACCATGGAGC ACTTTTTGCC	780
30	GCTGCGCAAC ATCTGGAACC GCGTCCGCGA CTTTCCGCGC GCCTCCACCA CCGCCGCCGG	840
	CATCACCTGG ATGTCCAGGT ACATCTACGG ATATCATCGC CTTATGTTGG AAGACCTCGC	900
	CCCCGGAGCC CCGGCCACCC TACGCTGGCC CCTCTACCGC CAGCCGCCGC CGCACTTTTT	960
35	GGTGGGATAT CAGTACCTGG TCGGGA CTG CAACGACTAC GTCTTTGACT CAAGGGCTTA	1020
	CTCGCGTCTC AGGTACACCG AGCTCTCGCA GCCGGGTAC CAGACCGTTA ACTGGTCGTT	1080
40	ATGGCCAACT GCAGCCCGGG GGATCCACTA GAAGAAGCTT GGGATCCGGC TGTGGAATGT	1140
	GTGTCAGTTA GGGTGTGGAA AGTCCCCAGG CTCCCCAGCA GGCAGAAGTA TGCAAAGCAT	1200
	GCATCTCAAC CAGACAGCAA CCATAGTCCC TCCCCTAACT CCGCCCATCC CGCCCCTAAC	1260
45	TCCGCCCAGT TCCGCCCATT CTCCGCCCCA TGGCTGACTA ATTTTTTTT TTTATGCAGA	1320
	GGCCGAGGCC GCCTCGGCCT CTGAGCTATT CCAGAAGTAG TGAGGAGGCT TTTTGGAGG	1380
50	CTGCCATCAT GGTTCGACCA TTGAACTGCA TCGTCGCCGT GTCCCAAAAT ATGGGGATTG	1440
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	TGACCACAAC CTCTTCAGTG GAAGGTAAAC AGAATCTGGT GATTATGGGT AGGAAAACCT	1560
55	GGTTCTCCAT TCCTGAGAAG AATCGACCTT TAAAGGACAG AATTAATATA GTTCTCAGTA	1620

- 55 -

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5 GCAGTTCTGT TTACCAGGAA GCCATGAATC AACCAGGCCA CCTCAGACTC TTTGTGACAA 1800
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10 CTAAGTTTGA AGTCTACGAG AAGAAAGACT AACAGGAAGA TGCTTTCAAG TTCTCTGCTC 1980
CCCTCCTAAA GCTATGCATT TTTATAAGAC CATGGGACTT TTGCTGGCTT TAGATCT 2037

15

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
20 (A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
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(ix) FEATURE:
(A) NAME/KEY: misc feature
(B) LOCATION: 1..22
30 (C) OTHER INFORMATION: /note= "VA1prim1"

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
40 (A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
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(ix) FEATURE:
(A) NAME/KEY: misc feature
(B) LOCATION: 1..23
50 (C) OTHER INFORMATION: /note= "VA1prim2"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

50

GGCACGCTTC AGCTGCACCC TTG 23

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What is claimed is:

- 1 1. An immortalized eukaryotic cell comprising
2 transfected DNA sequences operatively integrated into
3 its genome, said DNA sequences encoding:
4
5 (a) a viral transcription promoter operatively
6 associated with a DNA sequence encoding a morphogen,
7 said viral transcription promoter capable of being
8 stimulated by a viral transcription activator and
9 competent to act on and induce transcription of said
10 DNA encoding said morphogen to produce an RNA
11 transcript, said transcript, when translated, producing
12 a protein that, when properly folded, has a structural
13 conformation capable of inducing morphogenic activity
14 in a mammal;
15
16 (b) a transcription activator that acts on and
17 stimulates said transcription promoter; and,
18
19 (c) an RNA sequence operative to promote
20 translation of said RNA transcript.
- 1 2. The immortalized eukaryotic cell of claim 1 wherein
2 the copy number of said DNA sequence encoding said
3 morphogen and integrated into the genome of said cell
4 is less than 20 copies per cell.
- 1 3. The immortalized cell of claim 2 wherein the copy
2 number of said DNA sequence is less than 10 copies per
3 cell.
- 1 4. The immortalized cell of claim 3 wherein the copy
2 number of said DNA sequence is less than 5 copies per
3 cell.

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1 5. The immortalized eukaryotic cell of claim 1 wherein
2 said viral transcription promoter is selected from the
3 group consisting of promoters derived from adenovirus,
4 rous sarcoma virus, and cytomegalovirus.

1 6. The immortalized eukaryotic cell of claim 3 wherein
2 said viral transcription promoter comprises the
3 constitutive major intermediate early promoter of
4 cytomegalovirus.

1 7. The immortalized eukaryotic cell of claim 1 wherein
2 said transcription activator is a viral transactivating
3 protein competent to act on and stimulate transcription
4 of a non-homologous gene.

1 8. The immortalized eukaryotic cell of claim 7 wherein
2 said viral transcription activator is selected from the
3 group consisting of simian virus T antigen, adenovirus
4 E1A or E1B proteins, a protein encoded by the bovine
5 papillomavirus early region DNA sequence, and
6 herpesvirus IE proteins.

1 9. The immortalized eukaryotic cell of claim 8 wherein
2 said transcription activator is the adenovirus E1A
3 protein.

1 10. The immortalized eukaryotic cell of claim 1 wherein
2 said DNA sequence encoding said viral transcription
3 activator is operatively associated with a DNA sequence
4 defining a transcription promoter sequence competent to
5 act on and induce transcription of said transcription
6 activator DNA sequence, said transcription promoter DNA
7 sequence being selected to limit transcription of said
8 viral transcription activator DNA sequence.

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1 11. The immortalized eukaryotic cell of claim 1 wherein
2 said RNA sequence is a transactivating RNA sequence of
3 viral origin, competent to act on and enhance
4 translation of an RNA transcript encoded by a
5 heterologous gene.

1 12. The immortalized eukaryotic cell of claim 11
2 wherein said RNA sequence is selected from the group
3 consisting of the adenovirus VA1 RNA and an RNA
4 sequence encoded by the bovine papillomavirus early
5 region DNA sequence.

1 13. The immortalized eukaryotic cell of claim 12
2 wherein said RNA sequence is VA1.

1 14. The immortalized eukaryotic cell of claim 1 wherein
2 said cell, when cultured and harvested, produces at
3 least 1 μg of morphogen per 10^6 cells per ml culture
4 medium.

1 15. The immortalized eukaryotic cell of claim 14
2 wherein said cell produces at least 5 μg morphogen per
3 10^6 cells per ml culture medium.

1 16. The immortalized cell of claim 15 wherein said cell
2 produces at least 10 μg morphogen per 10^6 cells per ml
3 culture medium.

1 17. The immortalized cell of claim 14, 15 or 16 wherein
2 said protein is harvested when said cell is in post-
3 logarithmic phase.

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1 18. The immortalized eukaryotic cell of claim 1 wherein
2 said morphogen is a protein selected from the group
3 consisting of: OP1, OP2, OP3, BMP2, BMP3, BMP4, BMP5,
4 BMP6, BMP9, DPP, Vgl, Vgr, 60A protein, GDF-1, Dsl-1,
5 GDNF, and amino acid sequence variants thereof, wherein
6 said amino acid sequence variations do not alter
7 substantially the morphogenic activity of said protein.

1 19. The immortalized cell of claim 17 wherein said
2 morphogen is a protein selected from the group
3 consisting of OP1, OP2, OP3, BMP2, BMP4, BMP5, BMP6,
4 BMP9, and amino acid sequence variants thereof.

1 20. The immortalized eukaryotic cell of claim 1 wherein
2 said morphogen comprises an amino acid sequence having
3 at least 60% identity with the C-terminal 96 amino
4 acids of human OP1.

1 21. The immortalized eukaryotic cell of claim 1 wherein
2 said protein comprises a pair of polypeptide chains
3 associated to form a dimeric species.

1 22. The immortalized eukaryotic cells of claim 1
2 wherein said DNA sequence encoding said morphogen
3 encodes OP1 or an amino acid variant thereof, wherein
4 said amino acid sequence variation does not alter
5 substantially the morphogenic activity of said protein.

1 23. The immortalized eukaryotic cell of claim 1
2 comprising DNA sequences encoding at least two
3 morphogens, each said DNA sequence encoding a morphogen
4 being operatively associated with said viral
5 transcription promoter of claim 1.

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1 24. The immortalized eukaryotic cell of claim 22
2 wherein said DNA sequences are independently selected
3 from the group of DNA sequences encoding OP1, OP2, OP3,
4 BMP2, BMP4, BMP5, BMP6, BMP9, and amino acid sequence
5 variants thereof, wherein said amino acid sequence
6 variations do not alter substantially the morphogenic
7 activity of the protein.

1 25. The immortalized eukaryotic cell of claim 1, or 23
2 wherein said morphogen comprises a chimeric amino acid
3 sequence of two or more morphogens.

1 26. The immortalized eukaryotic cell of claim 1 wherein
2 said cell is a mammalian cell.

1 27. The immortalized cell of claim 24 wherein said cell
2 is a mammalian kidney, bladder, liver, lung, cardiac
3 muscle, or smooth muscle cell.

1 28. The immortalized eukaryotic cell of claim 25
2 selected from the group consisting of Chinese hamster
3 ovary cells, canine kidney cells, and rat bladder
4 cells.

1 29. The immortalized eukaryotic cell of claim 26
2 wherein said cell is a Chinese hamster ovary cell.

1 30. The immortalized eukaryotic cell of claim 1 further
2 comprising means for amplifying the copy number of said
3 DNA sequence encoding said morphogen.

1 31. The immortalized eukaryotic cell of claim 28
2 wherein said amplification means comprises a sequence
3 DNA encoding dihydrofolate reductase (DHFR) in
4 operative association with a promoter sequence that
5 acts on and induces transcription of said DHFR DNA.

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1 32. A method of producing a morphogen, said method
2 comprising the steps of:

3

4 (a) culturing the cell of claim 1 under conditions
5 sufficient to produce a population of said cells
6 expressing said morphogen, and

7

8 (b) isolating the morphogen produced by said
9 cells.

1 33. The method of claim 30 wherein said step of
2 culturing said cell comprises the step of growing said
3 cell in a serum-free medium comprising a lipid membrane
4 phosphoglyceride ester degradation product.

1 34. A method of manufacturing a cell line, the method
2 comprising the steps of:

3

4 (a) transfecting an immortalized eukaryotic cell
5 with a nucleic acid comprising a DNA sequence encoding
6 a viral transcription activator that acts on and
7 stimulates a viral promoter;

8

9 (b) transfecting said immortalized eukaryotic cell
10 with nucleic acids comprising DNA sequences encoding:

11

12 (1) a viral transcription promoter operatively
13 associated with a DNA sequence encoding a morphogen,
14 said viral transcription promoter capable of being
15 stimulated by said viral transcription activator, and
16 competent to act on and induce transcription of said
17 DNA sequence encoding said morphogen to produce an RNA
18 transcript, said transcript, when translated, producing
19 a protein that, when properly folded, has a structural
20 conformation capable of inducing morphogenic activity
21 in a mammal; and

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22
23 (2) an RNA sequence operative to promote
24 translation of said RNA transcript;
25
26 (c) culturing said transfected cell; and
27
28 (d) selecting a clone that expresses at least 1 μ g
29 morphogen/ 10^6 cell/ml.

1 35. The method of claim 32 wherein the transfecting
2 steps (a) and (b) are performed simultaneously.

1 36. The method of claim 32 wherein the transfecting
2 steps (a) and (b) are performed sequentially.

1 37. The method of claim 32 wherein said DNA sequence
2 encoding said viral transcription activator is stably
3 integrated into the genome of said cell before the
4 transfecting step of step (b).

1 38. The method of claim 32 wherein said DNA sequence
2 encoding said RNA sequence comprises part of a nucleic
3 acid separate and independent of a nucleic acid
4 comprising said DNA sequence encoding said viral
5 promoter operatively associated with said DNA sequence
6 encoding said morphogen.

1 39. The method of claims 32 or 36 wherein said nucleic
2 acids comprise independent vectors.

1 40. The method of manufacture of claim 32 wherein said
2 DNA sequences transfected in steps (a) and (b) are
3 stably integrated into the genome of the clone selected
4 in step (d).

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1 41. The method of manufacture of claim 32 wherein the
2 copy number of said DNA sequence encoding said
3 morphogen and integrated into the genome of said cell
4 is less than 20 copies per cell.

1 42. The method of manufacture of claim 39 wherein said
2 copy number of said DNA sequence is less than 10 copies
3 per cell.

1 43. The method of manufacture of claim 40 wherein said
2 copy number of said DNA sequence is less than 5 copies
3 per cell.

1 44. The method of manufacture of claim 32 wherein said
2 viral transcription promoter is selected from the group
3 consisting of promoters derived from adenovirus, rous
4 sarcoma virus, and cytomegalovirus.

1 45. The method of manufacture of claim 32 wherein said
2 viral transcription promoter comprises the constitutive
3 major intermediate early promoter of cytomegalovirus.

1 46. The method of manufacture of claim 32 wherein said
2 transcription activator is a viral transactivating
3 protein competent to act on and stimulate transcription
4 of a non-homologous gene.

1 47. The method of manufacture of claim 32 wherein said
2 viral transcription activator is selected from the
3 group consisting of simian virus T antigen, adenovirus
4 E1A or E1B protein, and herpesvirus IE proteins.

1 48. The method of manufacture of claim 32 wherein said
2 transcription activator is E1A.

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1 49. The method of manufacture of claim 32 wherein said
2 DNA sequence encoding said viral transcription
3 activator is operatively associated with a DNA sequence
4 defining a transcription promoter sequence competent to
5 act on and induce transcription of said transcription
6 activator DNA sequence, said transcription promoter DNA
7 sequence being selected to limit transcription of said
8 viral transcription activator DNA sequence.

1 50. The method of manufacture of claim 32 wherein said
2 RNA sequence operative to promote translation is a
3 transactivating RNA sequence of viral origin, competent
4 to act on and enhance translation of an RNA transcript
5 encoded by a non-homologous gene.

1 51. The method of manufacture of claim 32 wherein said
2 RNA sequence is selected from the group consisting of
3 the adenovirus VA1 RNA and an RNA sequence encoded by
4 the bovine papilloma virus early region DNA.

1 52. The method of manufacture of claim 48 wherein said
2 RNA sequence is VA1.

1 53. The method of manufacture of claim 32 wherein said
2 morphogen is a protein selected from the group
3 consisting of: OP1, OP2, OP3, BMP2, BMP3, BMP4, BMP5,
4 BMP6, BMP9, DPP, Vgl, Vgr, 60A protein, GDF-1, Dsl-1,
5 GDNF, and amino acid sequence variants thereof, wherein
6 said amino acid sequence variations do not alter
7 substantially the morphogenic activity of said protein.

1 54. The method of manufacture of claim 51 wherein said
2 morphogen is a protein selected from the group
3 consisting of OP1, OP2, OP3, BMP2, BMP4, BMP5, BMP6,
4 BMP9, and amino acid sequence variants thereof.

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1 55. The method of manufacture of claim 32 wherein said
2 morphogen comprises an amino acid sequence having at
3 least 60% identity with the C-terminal 96 amino acids
4 of human OP1.

1 56. The method of manufacture of claim 32 wherein said
2 protein comprises a pair of polypeptide chains
3 associated to form a dimeric species.

1 57. The method of manufacture of claim 32 wherein said
2 DNA sequence encoding said morphogen encodes OP1 or an
3 amino acid sequence variant thereof, wherein said amino
4 acid sequence variation does not alter substantially
5 the morphogenic activity of the protein.

1 58. The method of manufacture of claim 32 comprising
2 DNA sequences encoding at least two morphogens, each
3 said DNA sequence encoding a morphogen being
4 operatively associated with said viral transcription
5 promoter of claim 1.

1 59. The method of manufacture of claim 56 wherein said
2 DNA sequences are independently selected from the group
3 of DNA sequences encoding OP1, OP2, OP3, BMP2, BMP4,
4 BMP5, BMP6, BMP9, and amino acid sequence variants
5 thereof, wherein said amino acid sequence variations do
6 not alter substantially the morphogenic activity of the
7 protein.

1 60. The method of manufacture of claim 32, 50 or 53
2 wherein said morphogen comprises a chimeric amino acid
3 sequence of two or more morphogens.

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1 61. The method of manufacture of claim 32 wherein said
2 cell is a mammalian cell.

1 62. The method of manufacture of claim 59 wherein said
2 mammalian cell is a kidney, bladder, liver, lung, ovary
3 cardiac muscle or other smooth muscle cell.

1 63. The method of manufacture of claim 60 selected from
2 the group consisting of Chinese hamster ovary cells,
3 canine kidney, or rat bladder cells.

1 64. The method of manufacture of claim 61 wherein said
2 cell is a Chinese hamster ovary cell.

1 65. The method of manufacture of claim 32 further
2 comprising means for amplifying the copy number of said
3 DNA sequence encoding said morphogen.

1 66. The method of manufacture of claim 62 wherein said
2 amplification means comprises a sequence DNA encoding
3 dihydrofolate reductase (DHFR) in operative association
4 with a promoter sequence that acts on and induces
5 transcription of said DHFR DNA.

1 67. A method of producing a morphogen, said method
2 comprising the steps of:

3
4 (a) culturing the cell of claim 1 under conditions
5 sufficient to produce a population of said cells
6 expressing said morphogen, and

7
8 (b) isolating the morphogen produced by said
9 cells.

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1 68. The method of manufacture of claim 32 comprising
2 the additional steps of:

3

4 (a) culturing said clone under conditions
5 sufficient to produce a population of said clones
6 expressing said morphogen; and

7

8 (b) isolating said morphogen from the culture
9 media.

1 69. The method of claim 66 wherein said culture media
2 is a serum-free cell culture media comprising a lipid
3 membrane phosphoglyceride ester degradation product.

1 70. The method of claim 32 wherein said DNA sequences
2 encoding said transcription activator and said RNA
3 sequence occur on a single nucleic acid.

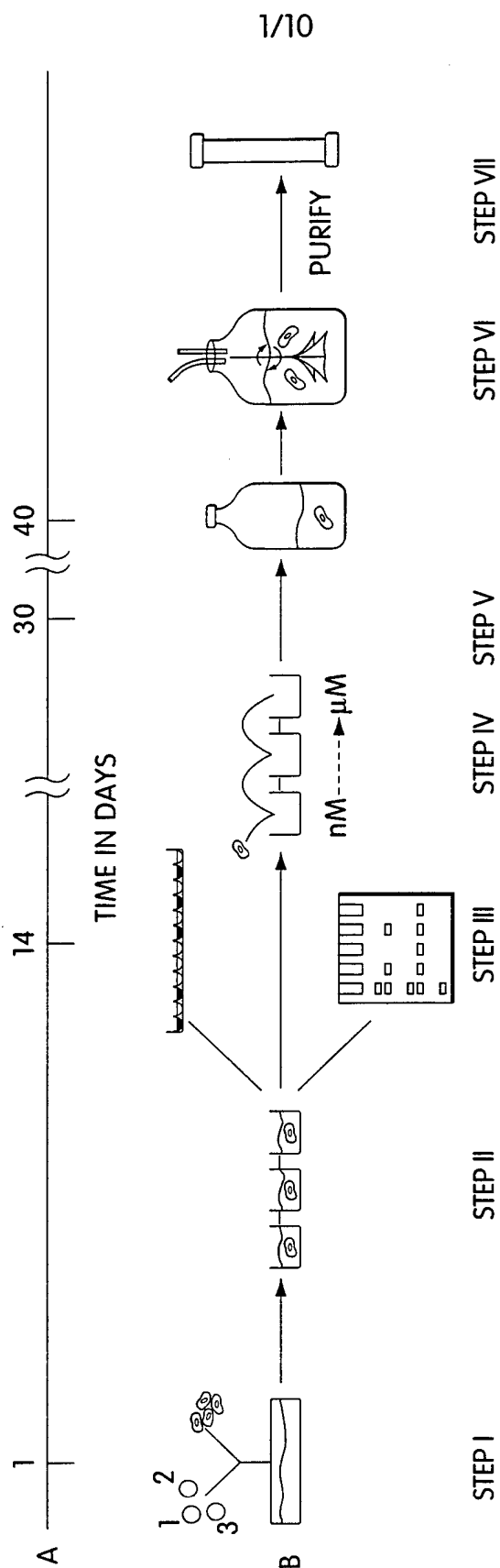


Fig. 1

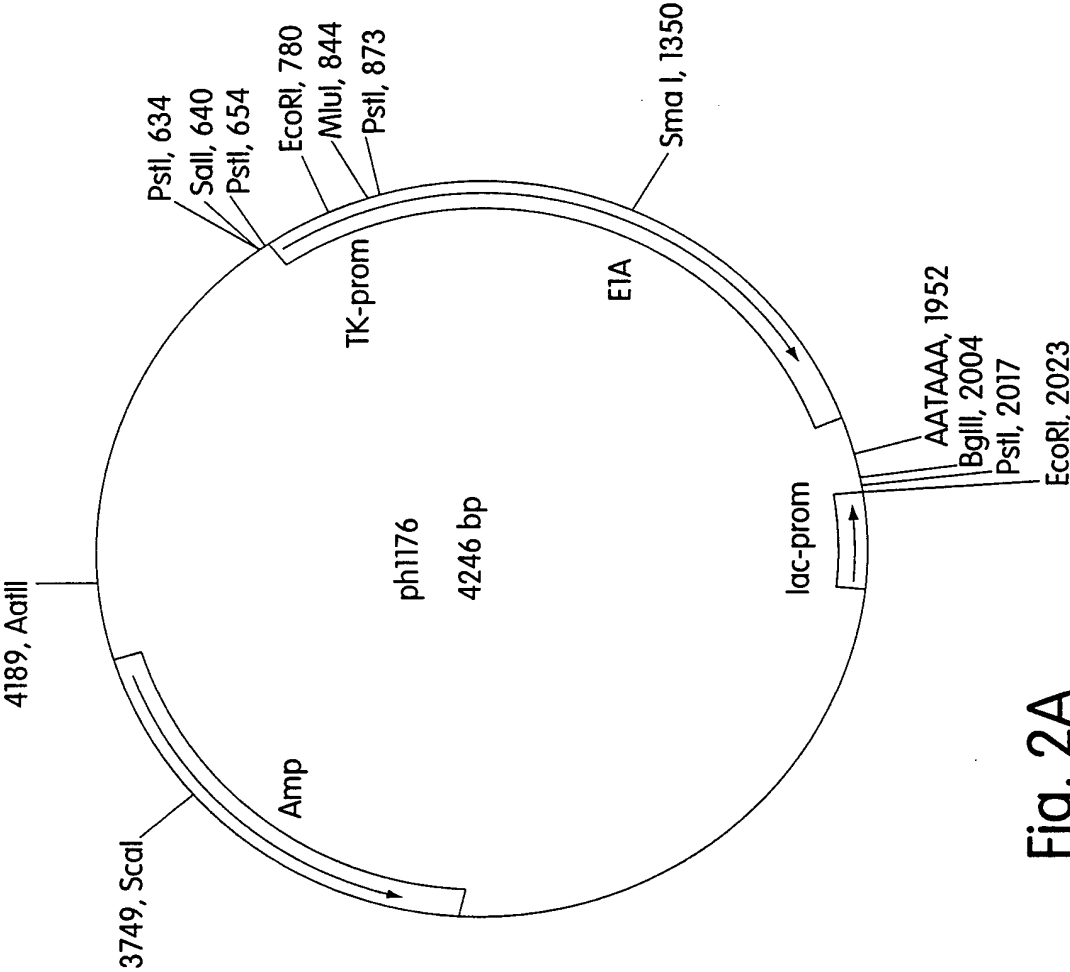


Fig. 2A

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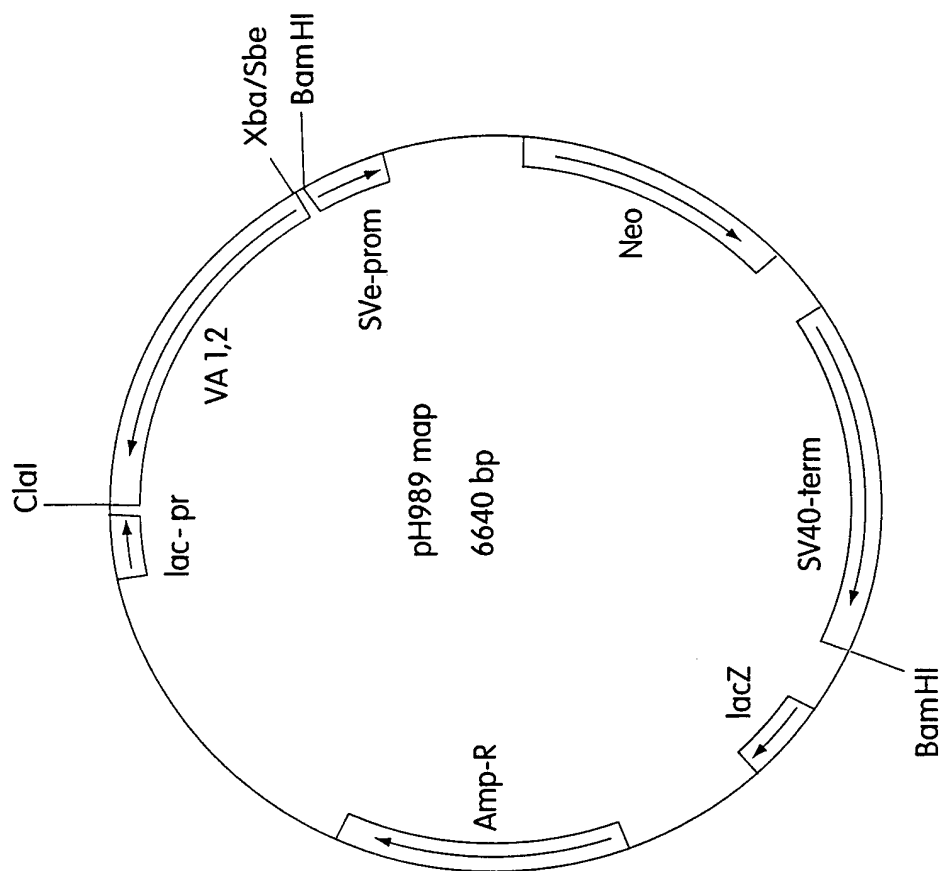


Fig. 2B

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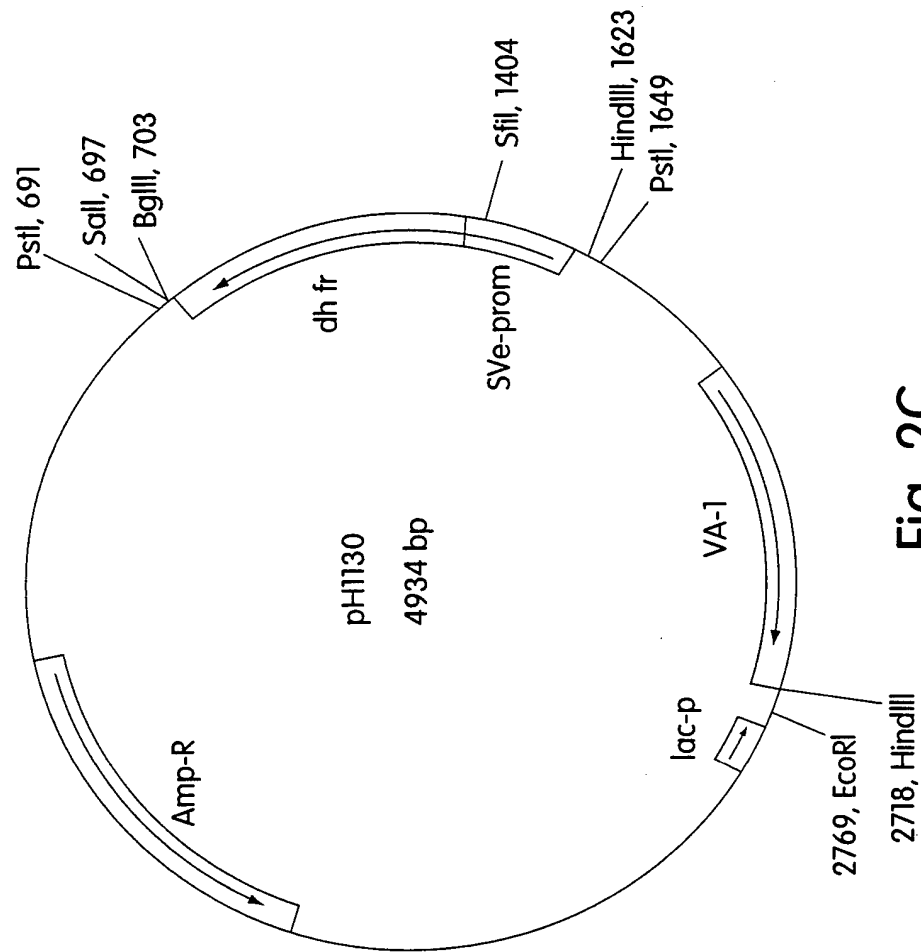


Fig. 2C

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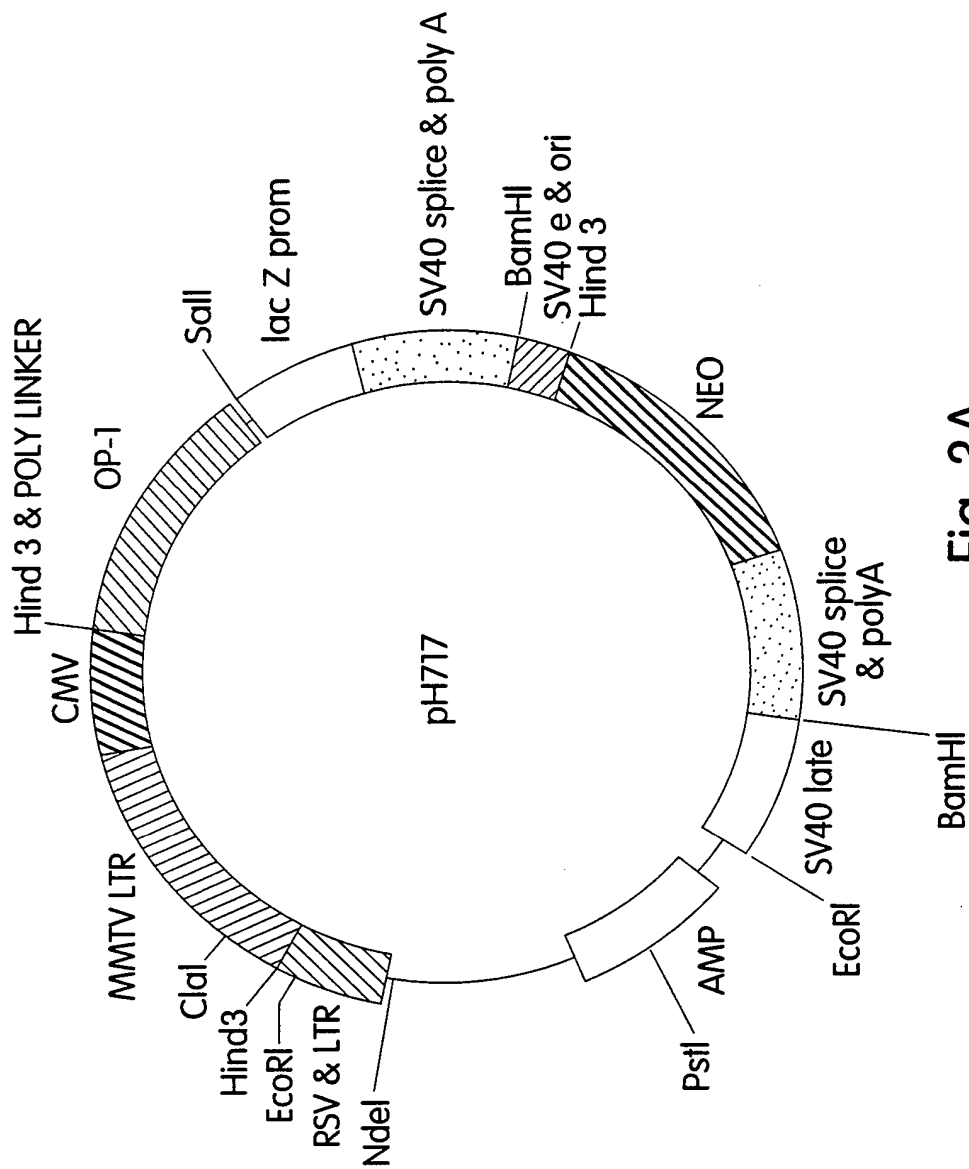


Fig. 3A

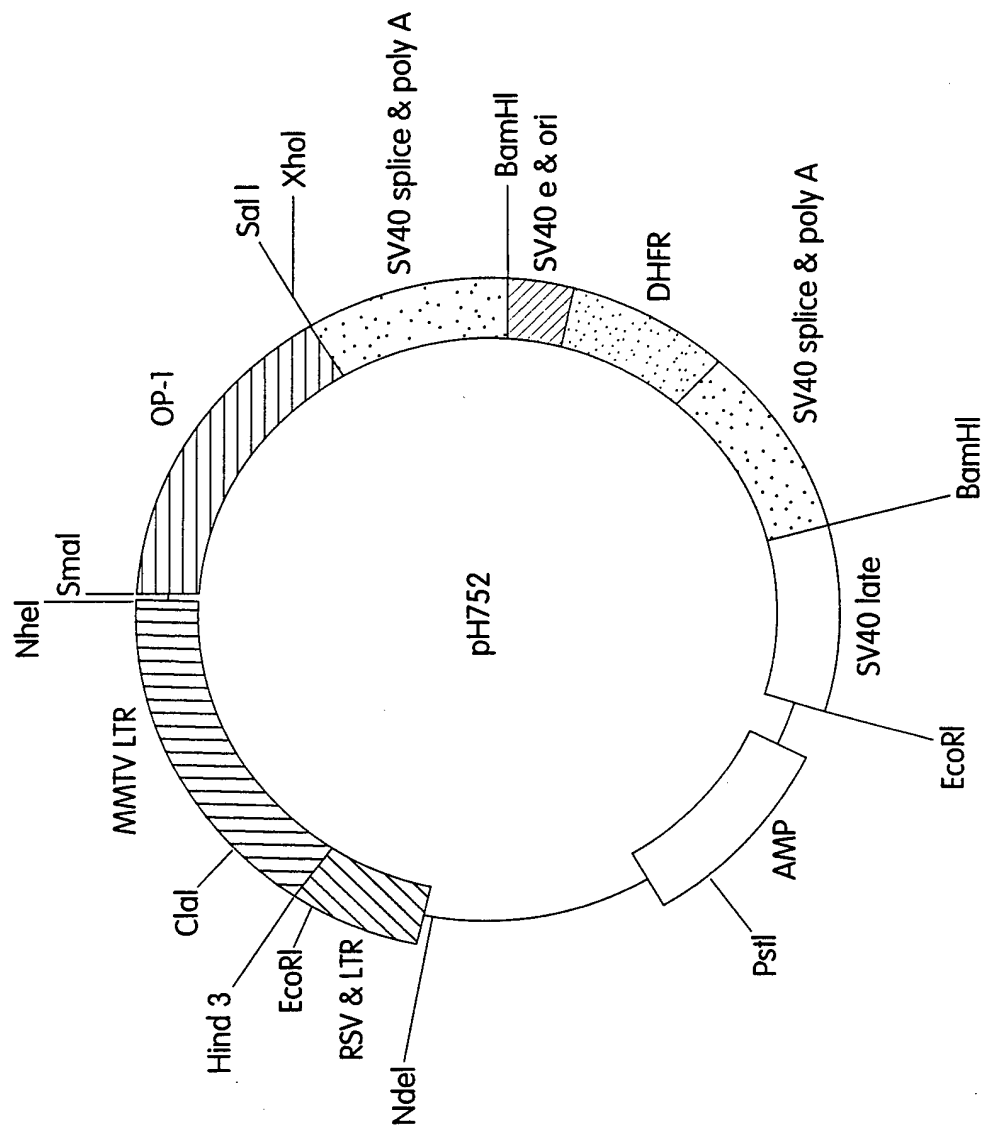


Fig. 3B

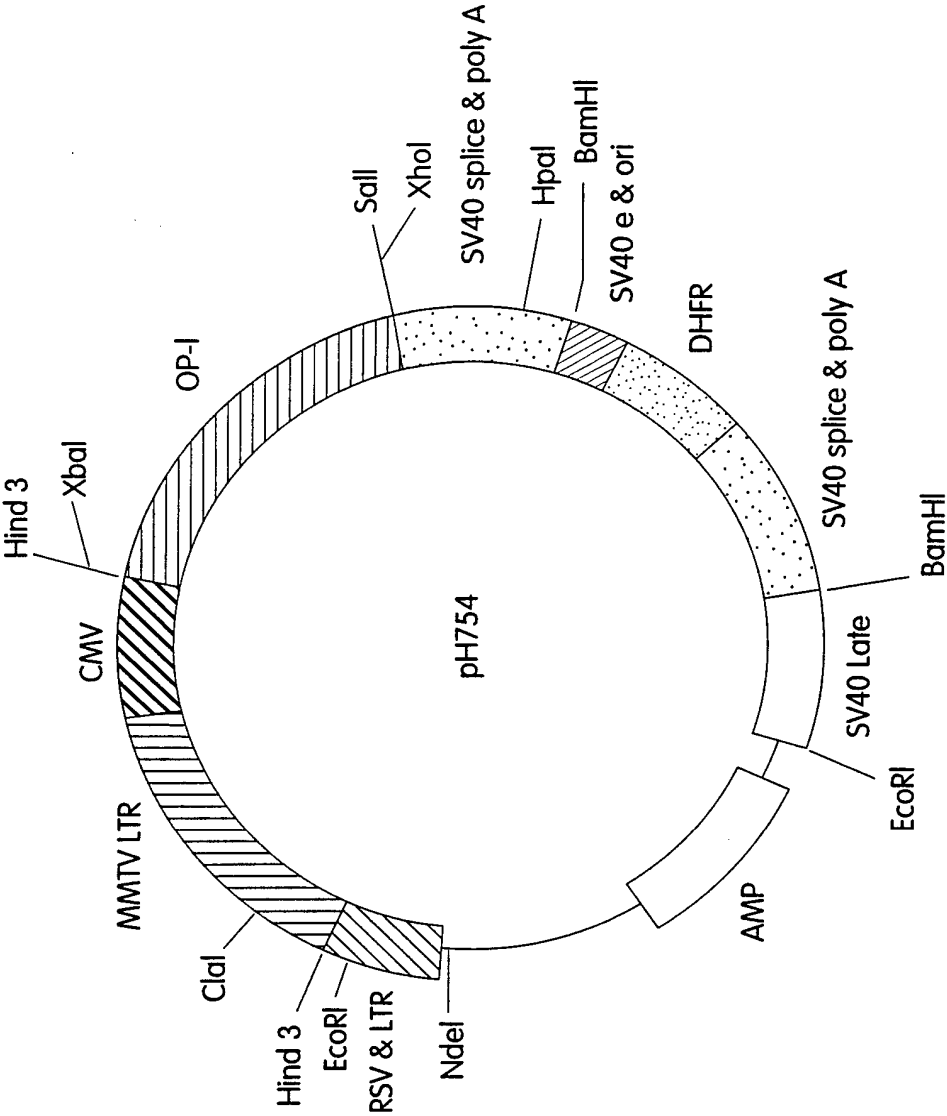


Fig. 3C

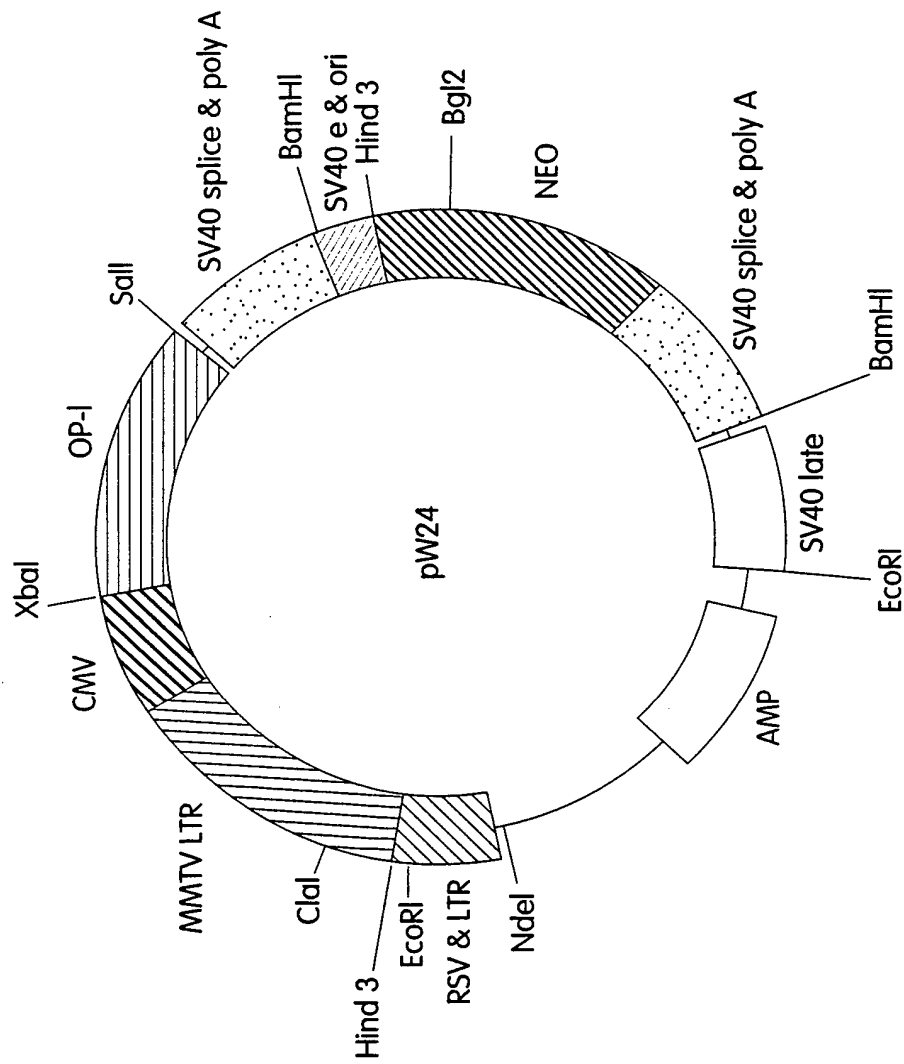


Fig. 3D

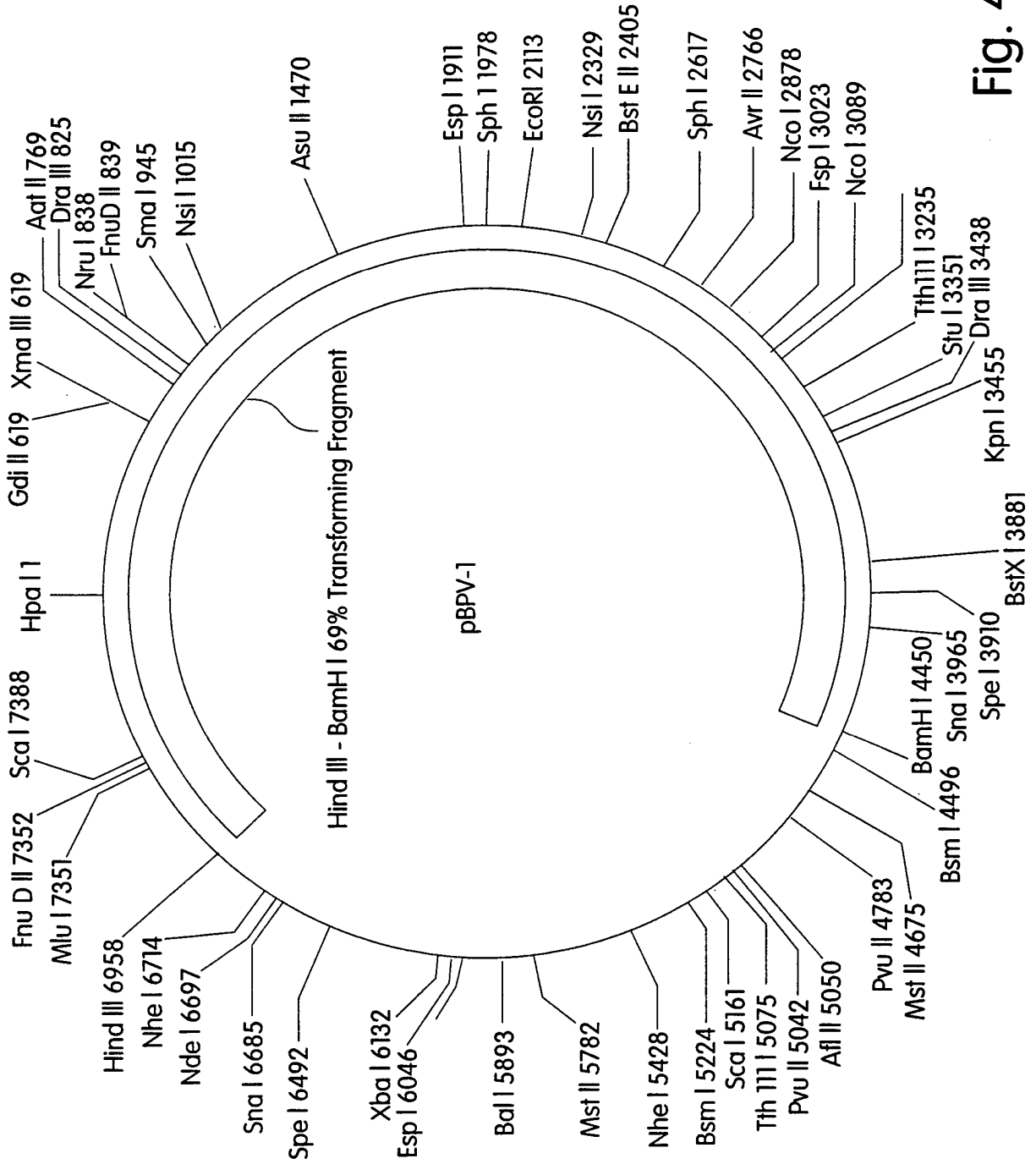


Fig. 4

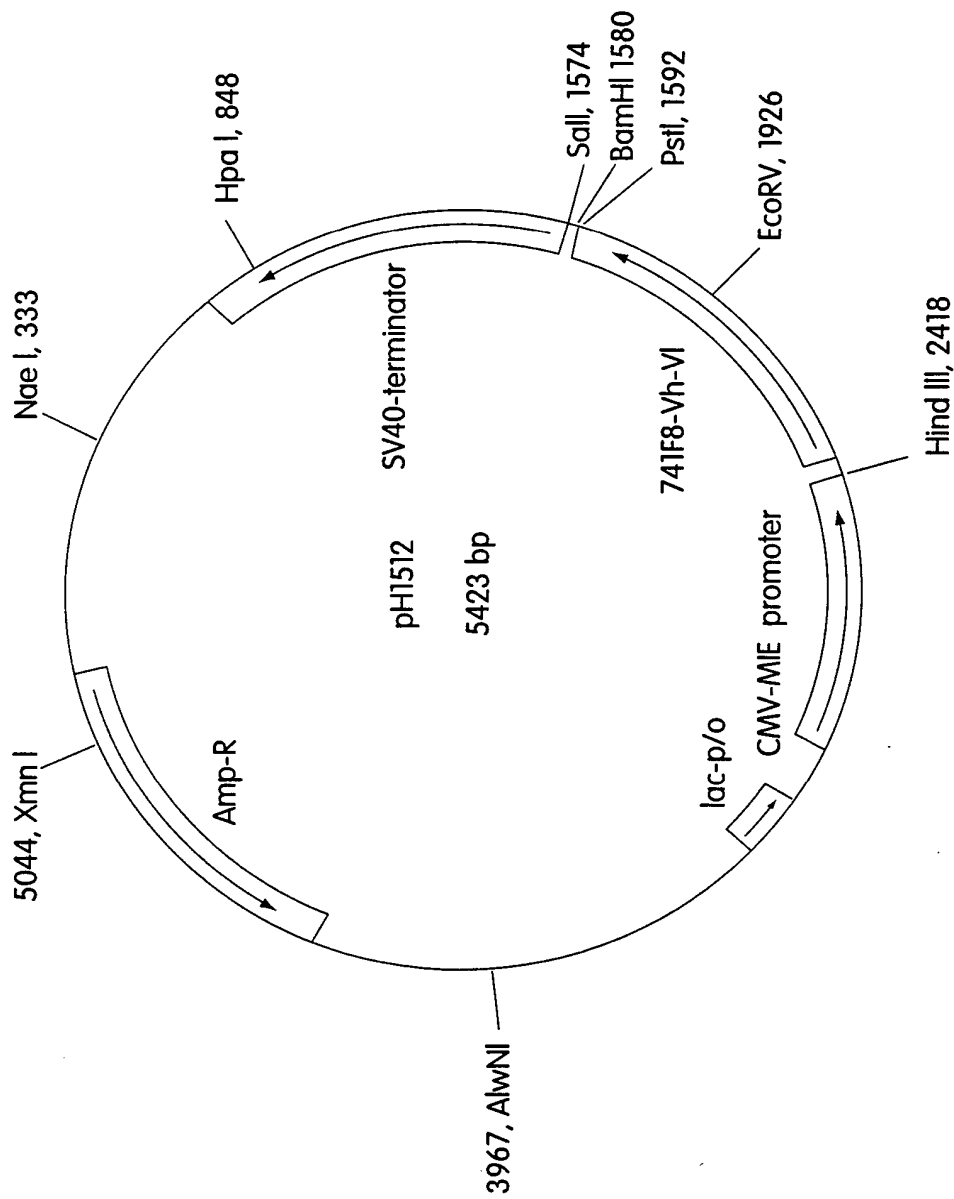


Fig. 5

INTERNATIONAL SEARCH REPORT

Intern Application No
PCT/US 94/12063

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/85 C12N5/10 C12N15/12 C07K14/51 C12N15/13
C07K16/28 C07K16/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP,A,0 309 237 (GENENTECH, INC.) 29 March 1989 cited in the application see page 3, line 35 - line 50 see examples 1,11 see claims	1-32, 34-68
Y	EP,A,0 378 382 (CELLTECH LIMITED) 18 July 1990 cited in the application see page 4, line 53 - page 6, line 44 --- -/--	1-17, 26-30, 32, 34-65, 67,68

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents :

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Date of the actual completion of the international search

31 January 1995

Date of mailing of the international search report

10.02.95

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Authorized officer

Andres, S

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/US 94/12063

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	SCIENCE, vol.228, 17 May 1985, LANCASTER, PA US pages 810 - 815 WONG, G. ET AL. 'Human GM-CSF: molecular cloning of the complementary DNA and purification of the natural and recombinant proteins' see figure 1 ---	30,31, 65,66
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